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The Radium Centre, Aarhus, Denmark (head: professor S. Kaae, M.D.) and
The Institute for Cancer Research, Aarhus, Denmark (head: J. Bichel, M.D.)

SOME INVESTIGATIONS ON THE MECHANISM OF THE LEUCOPENIA PRODUCED BY VINCOBLASTINE

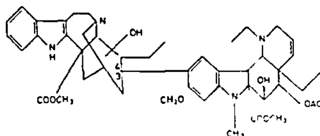
2. The Effect of Vincoblastine on Cells in Tissue Culture and in Vivo

By

J. Bichel

Received [illegible]

According to *Peckolt* (1910) an extract of the periwinkle Vinca rosea Linn. was used therapeutically early in this century by the Indians in Brazil against scurvy, toothache, persisting haemorrhage and slowly healing ulcers. However, as far as the author has found, no beneficial effect from this treatment has been reported. Furthermore, extracts of Vinca rosea have been used for diabetics in the Philippines and in Jamaica. The effect on the level of the blood sugar was studied simultaneously in the U.S. and in Canada, but no influence could be demonstrated. During those experiments it was found that the crude extract had an effect on the bone marrow and the white blood cell picture. In moderate doses it gave rise to a reversible leucopenia. Several alkaloids were then isolated. The first was vincoblastine (Velbe®, Lilly) (vinblastine sulphate) which was found to be a dimeric alkaloid containing both indole and dihydroindole moities (*Gorman et al.* 1959, Neuss et al. 1958). The structural formula was probably



CATHARANTINE [illegible]

Aided by grants from the *Anders Hasselbalch Anti-Leukaemia Foundation, Carl Shepler and Wife, Bequest,* the *Irma Foundation* and the *Danish Anti-Cancer League*.

Besides causing leucopenia vincoblastine also exerted an inhibiting effect on the growth of several animal tumours in mice and rats This was shown by *Cutts Beer & Noble* (1957) and later confirmed by many other investigators

The problems concerning the pathogenesis of the mechanism of the leucopenia have been outlined in the introduction to the first paper of this series Before the experimental investigations are described a brief report of what is known about the effect of vincoblastine on cells *in vivo* and *in vitro* will be given

It is generally agreed that in tissue culture and also *in vivo* vincoblastine acts on the cells as a typical spindle poison (*Hampel & Gerhartz* 1962 1964a 1964b *Cardinali et al* 1961 *Vaitkevicius* 1962 *Soldati & Gaetani* 1961 *Palmer et al* 1960 (cells from human monocytic leucemia) *Walker & Wright* 1962) Most authors describe the effect as completely similar to that of colchicine *i e* it produces metaphase arrest (c mitosis) A few authors are of the opinion that vincoblastine acts on the cell nuclei at an earlier stage of the cell division than colchicine Hampel & Gerhartz describe *e g* early prophase disturbances and also claim chromosomal changes to be different from those produced by colchicine According to those authors vincoblastine does not produce so pronounced exploded mitoses as colchicine and the pycnotic chromosomes have a greater tendency to fuse and form large pycnotic areas Cardinali et al mention that Cutts has not observed any inhibition in the prophase but they find the inhibition of prophase in human bone marrow as well as in leucemic cells from leucemia L 1210 In cells regenerated after vincoblastine influence the nucleus will often be abnormal multilobulated and now and then the cells may contain many nuclei

It is mentioned several times that the action of colchicine differs from that of vincoblastine *i a* that contrary to colchicine vincoblastine acts on the cells from the golden hamster (*Orsini et al* 1961) However this is probably not quite correct as colchicine acts on hamster cells as a spindle poison but the concentrations must be rather high

Besides being a spindle poison vincoblastine is also an antimetabolite as several of its effects i a its action on the spindle may be prevented by certain amino acids as glutamic acid tryptophan *α* keto glutaric acid and also by Co enzyme A (*Johnson et al 1960*) *It must* however be remembered that the effect of colchicine also may be prevented at least by meso inositol (*Murray et al* 1951)

The effect of vincoblastine seems to be the same *in vivo* and *in vitro* anyway as far as morphological alterations are concerned Injections of vincoblastine to normal animals (mice rats dogs hamsters) will produce a mitotic arrest This is most pronounced in the granulocytopoietic tissue in the bone marrow especially in the myelocytes

Some of the most extensive studies of the effect of vincoblastine on cells in tissue culture of bone marrow and also of the effect on cells

from leucemia L 1210 and Ehrlich ascites tumours have been done by *Cutts* (1961). He found that after a single injection of vincoblastine the number of mitoses increased progressively and reached a maximum after 12 hours whereafter the number decreased. After 48 hours the number of metaphases was still somewhat increased. In tumours the number of mitoses decreased to normal values more rapidly than in normal tissue. The arrested mitoses occurred very soon after the injection and in most animals they reached a peak in the 12th to the 16th hour in some animals already in the 8th hour. The reconstruction of the nuclei might result in deformities of the nucleus as described by *Cardinali et al.* (1961) but also in giant nuclei. During that process or during the production of multilobulated nuclei an exclusion of one or more chromosomes might occur. Cutts did not observe chromosome breaks as those regularly produced by alkylating agents. Cutts also demonstrated that the administration of glutamic acid just prior to vincoblastine reduced the number of arrested mitoses. His investigations were done on Swiss mice bearing an ascites tumour. Cutts compared the effect of colchicine and vincoblastine and although he emphasized that vincoblastine is a typical c-mitotic poison the degree of inhibition was more lasting and complete than that produced by colchicine. According to *Cutts* a weak concentration of vincoblastine will inhibit the formation of the spindle while strong concentrations also affect the chromosomes directly.

Vaitkevicius (1962) found that the mitotic arrest will appear in tumours which may be inhibited by vincoblastine as well as in vincoblastine resistant tumours. Consequently it is hardly this effect which causes tumour inhibition.

In conclusion it will appear that vincoblastine is a spindle poison which probably does not act quite like colchicine. It is also stated that vincoblastine is an antimetabolite as several of its effects may be prevented by different amino acids.

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MATERIAL AND OBSERVATIONS

Between 1955 and 1962 the Department of Pathology of the I and II Clinics of Obstetrics and Gynaecology Helsinki University Central Hospital examined a total of 23 histological specimens which could be diagnosed as mesonephric carcinoma 12 of the specimens of the present material were collected from the clinics referred to while 11 specimens were sent in by provincial hospitals Supplementary information on the patients was obtained from the attending doctors and the patients themselves

The histological specimens were fixed in 10 per cent formalin for one to three days and cut with a microtome into sections 5 to 6 µ thick The sections were stained by the haematoxylin van Gieson method As the purpose of the investigation was only to show the diagnostic possibilities by the aid of common routine histologic examination no attempt to differentiate the varying types of carcinoma by means of special staining methods was performed in this connection

The average age of the patients concerned was 51 years the oldest being 84 and the youngest 36 years old A more detailed presentation of the cases is given in Table 1 In 20 cases the tumour was situated in the ovary in 2 in the cervix uteri and in 1 in the wall of the vagina In the literature only 81 cases have been reported in which the mesonephric carcinoma was situated in the area of the cervis uteri (1 and 11) Mesonephric carcinoma with the exception of cyst like forms is also rare in the area of the vagina (1)

When microscopic preparations made from the tumour specimens were examined attention was paid not only to the characteristic clear cell changes (Figs 1 2 3) but also to structures denoting a possible connection with the serous cystadenoma tumour type (Fig 4) as well as to endometrioid traits (Fig 5) Besides the clear cell pattern distinct papillary structures were observed in 12 cases smaller cystic formations in 11 cases and epithelium of serous type in 7 cases In 3 cases the endometrioid structure predominated (cases 10 16 and 20) and in 5 cases endometrioid structures occurred alongside clear cell changes (cases 5 9 13 14 and 18)

Table 1 also gives the methods and results of treatment In all but three cases the treatment was operative In most cases surgery was supplemented with X ray therapy Two cases were treated with radio active gold and in only one case were cytostatics given In two cases X ray therapy alone was given and in one (case 12) the patient refused all treatment Four patients have survived five years without recurrence (case 4 11 15 and 22) one (case 19) two and a half years and one (case 23) one and a half years One patient died as the result of an accident two years and 10 months after treatment (case 7) and one patient (case 17) died 4 years and 8 months after treatment the cause of death being carcinoma of the brest



Fig [illegible]

Fig 1 Clear cell [illegible] r in oma with papill [illegible] t [illegible] pr l f [illegible] ti [illegible] (a e [illegible] H m + van Cieson × [illegible]00

Fig [illegible] Adenom [illegible] t u [illegible] re [illegible] of clear c ll [illegible] m ([illegible] 7 H m [illegible] n Cie n × [illegible]00

TABLE 1

Summary of 23 Clear Cell Carcinoma

No	Age	Localis of tumor	Histol type of tumor beside clear cell structure		Methods of treatment	Follow up
1	45	both ovaries	serous cystic	+ tcc	hysterect bil s o ect x ray	died in 4 mos
2	[illegible]	ovary + ca corp ut	papill	+ tcc	Ra appl x ray	died in 13 mos
3	39	ovary	serous cystic papill	+ tcc	sv amput bil s o ect x ray Au	died in 41 mos
4	49	ovary	serous cystic papill	+ tcc	sv amput bil s o ect x ray	alive (7 years)
5	51	ovary	endometr	+ tcc	sv amput bil s o ect x ray Sendoxan	died in 9 mos
6	55	ovary	serous cystic papill	+ tcc	sv amput bil s o ect x ray	died in 34 mos
7	40	ovary	serous cystic papill	+ tcc	sv amput bil s o ect x ray	died in 34 mos
8	36	ovary	papill endometr	+ tcc	sv amput bil s o ect Au	died in 4 mos
9	54	ovary	cystic papill endometr	+ tcc	hysterect bil s o ect x ray	died in 14 mos
10	61	ovary	endometr	+ tcc	hysterect bil s o ect x ray	died in 16 mos
11	48	ovary	serous cystic papill	+ tcc	hysterect bil s o ect x ray	alive (9 years)
12	84	cervix uteri	serous cystic papill	+ tcc	nothing	died in 12 mos
13	36	ovary + ca recti	cystic endometr	+ tcc	s o ect sin x ray	died in 9 mos
14	47	ovary	cystic endometr	+ tcc	s o ect dx x ray	died in 5 mos
15	50	ovary	papill	+ tcc	hysterect bil s o ect x ray	alive (8 years)
16	39	vagina	endometr	+ tcc	exstirp cysta vagin	died in 8 mos
17	56	cervix uteri + ca mammae	solid clear cell ca		Ra appl x ray	died in 56 mos
18	44	ovary	endometr	+ tcc	hysterect bil s o ect Ra appl x ray	died in 3 mos
19	77	ovary		+ tcc	sv amput bil s o ect x ray	alive (2 ½ years)
20	36	ovary	endometr		sv amput bil s o ect	died in 3 mos
21	62	ovary	cystic		hysterect bil s o ect x ray	died in 3 mos
22	56	ovary	endometr papill	+ tcc	sv amput bil s o ect x ray	alive (8 years)
23	69	ovary		tcc	sv amput bil s o ect x ray	alive (1½ years)

tcc — typical clear cell carcinoma



Fig 3

Predominantly solid part of clear cell carcinoma, case 19. Haem + van Gieson × 200

DISCUSSION

The tumours of the present series are all of clear cell type, to which the term mesonephric carcinoma possibly could be applied (9).

Our cases 12, 16 and 17 are indisputable insofar that besides being of characteristic histological structure they originate from paravaginal tissue, *i.e.* from an area in which there can hardly be any question of another type of histogenesis.

With regard to the ovarian tumours, however, the histogenesis is perhaps not so firmly established. For, besides tumours arising from mesonephric vestiges, there occur in the ovaries two other forms of carcinoma which may also be of clear cell type, and distinguish between these and true mesonephric carcinoma may thus prove difficult and even impossible. One of these carcinomas is the malignant serous papillary cystadenoma. Though our series does not comprise a single *grossly cystic ovarian tumour, multiple* smaller cystic *formations were* found in several cases, as well as histological features pointing towards this type of tumour. Another tumour which may occasionally occur either partly or entirely as a clear cell type tumour is adenocarcinoma originating from ovarian endometriosis. It has been established that of this endometrioid carcinoma a clear cell type variant may occur ( ).
It is highly probable that endometrioid carcinoma and serous cystade

Figs 4–5

Fig 4 Structures typical of adenofibroma Case 7 Haem + van Gieson × *100*
Fig 5 Carcinoma with partly endometrioid partly clear cell structures Haem + van Gieson × 150

noma of the ovary are histogenetically closely related (4). Thus our series includes cases ([illegible], 9, 10, 13, 14) in which areas of endometrioid type of varying size were seen. Consequently in our opinion, which agrees with the statements of *Willis* (10) and *Haynes & Taylor* (2), a definite histogenetic diagnosis with regard to the clear cell carcinoma occurring in the ovary cannot be made for the present. Without disputing the possibility that ovarian tumours of purely clear cell type may actually originate from mesonephric remnants, we still think that the two above mentioned possibilities must be considered.

Our present knowledge does not allow us in anywhere near all cases to determine with full certainty the histogenesis of the ovarian carcinomas in question.

SUMMARY

The authors present 23 mesonephric carcinomas, 20 of which were situated in the ovary, 2 in the cervix uteri and 1 in the wall of the vagina. With regard to the histogenesis of ovarian carcinoma there is at any rate in some cases some uncertainty, since malignant serous cystadenoma papilliferum and endometrioid carcinoma may occur as clear cell types.

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EXPERIMENTAL INVESTIGATION ON INFLUENCE OF HYDROCORTISONE ON SPREAD OF TRANSPLANTED ROUS RAT SARCOMA IN SYNGENEIC TUMOUR HOST SYSTEM

By

BENGT KALLUM and TOM SALDEEN

Received 18 x 66

Experimental investigations on the effect of exogenous factors on tumour spread have given controversial results which may be due to differences in methods of treatment but also to host factors or unknown intrinsic characteristics of the various tumours used (Surveys *Cole et al* 1961 *Nielsen* 1962)

In an earlier investigation it was shown that hydrocortisone stimulated the spread of the transplantable Rous rat sarcoma in an allogenic tumour host system (*Saldeen* 1963) The purpose of the present investigation was to study the effect of hydrocortisone on the spread of the rat sarcoma under the same experimental conditions in a syngeneic tumour host system

MATERIAL AND METHODS

Animals—White rats of a strain inbred since several years were used It was obtained from Dr C M *Rudenstam* Gothenburg and during the experimental period 8 months kept in our institute by brother sister mating

Skin grafting between them at least 1 month old rats was performed using a method described by *Thomas et al* (1961) As controls autografts and allografts were used About one month after the operation all the syngeneic grafts and autografts were fur bearing while the allografts had disappeared No signs of adverse reactions or breakdown were observed in the syngeneic grafts Still 20 weeks later the grafts were distinguishable from surrounding recipient skin only by the direction of the *hair follicles*

Tumour The origin of Rous rat sarcoma and the growth and spread of the transplanted sarcoma has earlier been described (*Ahlström & Jonsson* 1962 *Saldeen* 1962 1963) In the present investigation 2 tumour strains were used (Fig 1)

Strain 1—A series of inbred newborn rats were inoculated subcutaneously with 0 1 ml cell free tumour material from a chicken with Rous chicken sarcoma (Strain S R) After an interval of 21 days all the rats had cysts in the axillae and groins and after 36 days 4 of the 8 inoculated rats had palpable tumours at the site of injection Five of the rats were killed 3 months after the injection All had tumours and cysts 4 had lymph node metastases and 3 showed lung metastases One of the tumours was transplanted subcutaneously to a series of newborn rats Takes were obtained after 3 weeks and the tumour has since been carried serially in at most 7 day old rats (Fig 1) Tumour material from the first and third passages were used *for the experiments*

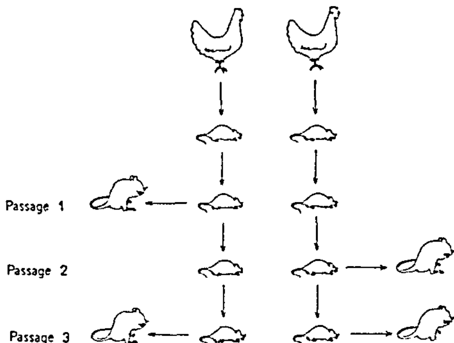


Fig 1
Transplantation scheme *Small rats* = rats for serial transplantation
Big rats = rats for the experiments.

Strain ? —The second tumour originated from a series of inbred newborn rat inoculated subcutaneously with 0 1 ml cell free chicken tumour material After 14 days all 10 rats had palpable tumour and were killed after 50 days. Two of them had lymph node metastases and one lung metastase One of the tumour was transplanted to 7 newborn rat They were killed after 16 days when 6 had tumours, 2 with lung metastases. The tumour was then serially transplanted in a m t 4 day old rats (Fig 1) Tumour material from the second and third passage of this strain were used for the experiments

Growth and appearance of the transplantable tumour—The growth and the histological appearence of the tumour did not differ from that earlier described in allogeneic tumour host system (*Ahlstrom & Jonsson 1962 Sjogren 1962 1963*) The individual variations were not less in syngeneic than in allogeneic system

Preparation of tumour suspension—A slight modification of a trypsin DNAase method described by *Madden & Burk* (1961) was used The tumour cells were counted in a Buerker haemocytometer after dilution 1 100 in physiological saline containing 0.0 per cent eosin Only unstained ("living") cells were counted.

RESULTS

Intramuscular tumours in the thigh The material consisted of 48 female rats weighing about 175 g and divided into 2 equal groups They were inoculated intramuscularly in the left thigh with 10^7 living tumour cells From 2 days before the injection of tumour until the end of the experiment 24 of the rats were treated with 2.5 mg hydrocortisone acetate (Hydrocortil Pharmacia) subcutaneously in the right thigh while the other 24 rats were treated with NaCl The rats were killed

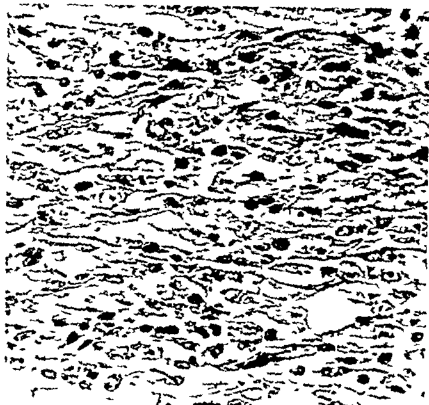


Fig 2 a
Rous rat sarcoma. Untreated rats

3 weeks after the tumour injection All had tumours at the site of injection The average tumour volumes (V = 0 5236 d³ d mean diameter of the tumour) were 25 3 cm³ *for the hydrocortisone* treated rats and 23 8 cm³ for the controls The *difference* was not *significant* The average carcass weight (without tumour) was 180 g in the hydrocortisone group 235 g in the control group The difference was significant No certain histological differences could be found in the structure of the primary tumours in the 2 groups In 6 of the *hydrocortisone* treated rats however there was a slight tendency towards disintegration of the stroma with loosening of the tumour cells (Fig 2)

One of the control rats showed metastases in the lumbar lymph nodes whereas 7 of the hydrocortisone treated rats had metastases in the regional nodes (lumbar or inguinal) Three other hydrocortisone rats showed metastases also in *more remote* lymph nodes (renal external or axillar) and in the lungs

Treatment with hydrocortisone thus resulted in an increased spread of the intramuscular tumours in the thigh to lymph nodes and lungs

Intravenous injection of tumour cells—Nineteen female rats weighing about 200 g were inoculated intravenously into a tail vein with

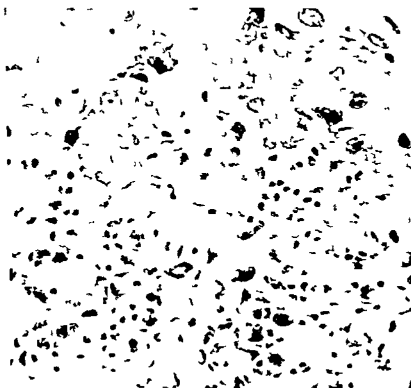


Fig 9 b
Rous rat sarcoma Hydrocortisone treated rat Disintegration of stroma and loosening of tumour cells.

5 × 10 living tumour cells Eight of them were treated on 2 days before the injection of the tumour and throughout the experiment with 2 5 mg hydrocortisone daily The other 11 rats served as controls The rats were killed on the 15th day after the tumour injection The weight of the control rats varied between 220–250 g (average 230 g) that of the hydrocortisone treated animals between 160–190 g (average 170 g)

Seven of the hydrocortisone treated rats and one of the controls had widespread pulmonary tumours whereas the other rats showed no pulmonary tumours No rats had tumours outside the lungs The pulmonary tumours often showed large central necroses

Treatment with hydrocortisone thus resulted in an increased frequency of metastases in the lungs following intravenous injection of tumour cells

DISCUSSION

There was no general difference in respect of growth and spread of Rous rat sarcoma in untreated rats in syngeneic tumour host system compared with that in allogeneic system

As to the effect of hydrocortisone in the allogeneic system (*Saldeen* 1963) 18 of the 19 treated rats against 2 of the 18 controls had lung metastases after intravenous injection of tumour cells In rats with intramuscular tumours 1 of 10 had metastases to the regional lymph nodes only while the other 9 rats had metastases to more remote lymph nodes also One of 10 control rats had metastases in regional lymph nodes

A comparison of the effect of hydrocortisone on the spread of allo geneic (*Saldeen* 1963) and syngeneic Rous rat sarcoma thus speaks for hydrocortisone having almost identical effect in the 2 systems after intravenous injection of tumour cells but a more pronounced effect in the allogeneic system than in the syngeneic on the spread of intra muscular tumours This may be due to small differences (not morpho logically verified) between the tumour strains used in the two experi ments *e g* in the present investigation only very early tumour passages were used It seems not probable that the hydrocortisone effect in the allogeneic system occurred via an immunological enhancement since *Kaliss et al* (1954) found no effect of cortisone on metastases of allo geneic tumours in mice subjected to immunological enhancement

Also in the syngeneic Rous rat sarcoma system there are lack of complete genetical identity between tumour and host since Rous sar coma of the strain used possesses common tumour specific antigen(s) (*Jonsson* 1966)

The hydrocortisone may possibly act by modifying the discrepancy between tumour and host

However it seems probable that hydrocortisone also has a direct effect on the tumour spread in addition to its possible indirect effect via the influence on tumour host relationship in transplantable systems It have been shown earlier that cortisone promotes the spread of human tumours (*Iversen & Hjort* 1958 *Hartman & Sherlock* 1961)

There are at least seven possible points of attack of an exogenous factor on the lymphatic spread (Fig 3) The exogenous agent may act on

1) Primary tumour *e g* by altering the mutual cohesiveness
2) Tumour host interzone *e g* by modifying the stroma reaction
3) Individual cancer cells *e g* by changing ameboid activity or sur face properties
4) Vascular invasion *e g* by modifying capillary permeability
5) Vascular endothelium *e g* by altering the endothelial stickiness (*Zeidman* 1962)
6) Lymph *e g* by changing the coagulability
7) R E S *e g* by modifying the phagocytic activity of the reticular cells in the cortical sinus

The present investigation speaks in favour of hydrocortisone acting on the first (Fig 2 b) point Hydrocortisone is however also known to

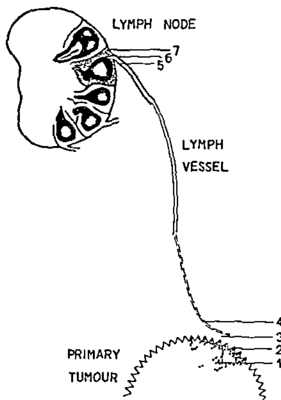


Fig 3
Possible points of attack of an exogenous factor on lymphatic spread See text

reduce the function of R E S and treatment by this drug results in involution of the lymphatic tissue It thus probably also promotes spread by acting on the last point

No histological differences were seen in the tumour host zone between hydrocortisone treated rats and controls Neither were any differences seen between the morphological picture of individual cancer cells in the lymph nodes of cortisone treated rats and controls This however of course does not exclude that hydrocortisone may modify the properties of the cells

Hydrocortisone is not known to increase capillary permeability

The haematogenic spread of the tumour may have been influenced by the effect of hydrocortisone on the stickiness of the vascular endothelium (*Zeidman* 1962) or the blood coagulability in the pulmonary vessels Tumour cells injected intravenously into a tail vein can pass the lungs in untreated animals (*Saldeen* 1963) and are possibly trapped to a greater percentage in the lungs of hydrocortisone treated rats As almost all tumour cells are trapped by the lymph node sinus already in untreated rats possible similar effect (*Saldeen* 1963) of hydrocortisone

on stickiness and lymph coagulability in the lymph node sinus are probably of minor importance

SUMMARY

Treatment with hydrocortisone promoted tumour spread in rats with early passages of transplantable Rous rat sarcoma in a highly inbred brother sister mater strain It seems most probable that hydrocortisone promotes the lymphatic spread of the tumour by acting on the primary tumour and on the R E S

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EFFECTS OF NITROGEN MUSTARD ON THE DEVELOPMENT OF EXPERIMENTAL AMYLOIDOSIS IN MICE

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Received 5.xi 66

An accelerating effect of nitrogen mustard on the formation of amyloid deposits was demonstrated by *Teilum* in 1954 In his experiments mice previously treated with casein for four weeks rapidly developed a wide spread amyloidosis after three doses of 2 5–5 mg/kg nitrogen mustard each second day while no or only a slight increase occurred in the controls Earlier investigations by the same author had showed a similar accelerating effect of cortisone and ACTH (*Teilum* 1952) In the same way x irradiation has been shown to be able to promote amyloid formation in mice (*Christensen & Hjort* 1959 1960) A common feature of all these experiments was the observation that an acceleration of the amyloidogenic process depended on the time of administration of the proper accelerating agent Only when applied late in the experiment—that is near the end of the primary pyronino philic phase or in the second amyloid phase could acceleration be constantly achieved On the other hand when cortisone was given before or simultaneously with the amyloidogenic treatment—that is during the initial pyroninophilic phase formation of amyloid in mice was inhibited or in some cases even prevented in spite of 6 weeks of casein treatment This inhibition was found to involve a suppression of the cellular proliferations showing cytoplasmic pyroninophilia (*Christensen* 1961) These seemingly contradictory effects of cortisone support the two phase theory of local amyloid formation (*Teilum* 1964) and it has been anticipated that other agents may exhibit the same pattern of inhibition acceleration depending on dose and time of administration during amyloid induction Recently heterologous antilymphocytic serum has proved effective in preventing amyloid formation in casein treated mice when applied during the initial pyroninophilic phase (*Ranlov* 1967) In the following the effects of nitrogen mustard on amyloid formation in casein treated mice will be shown to vary depending on the time of administration

This investigation was supported by *The Danish League Against Rheumatism*

MATERIAL AND METHODS

ST/a mice of equal sex distribution 3–4 months of age were used after randomization. They were divided as follows.

Group I

15 mice were treated for 4 weeks with daily subcutaneous injections 5 times a week of 0.5 ml 5 per cent sodium caseinate in 0.25 per cent NaOH—a total of 20 casein injections. Simultaneously nitrogen mustard was administered every second day (monday–wednesday–friday) for 4 weeks 0.02 mg nitrogen mustard (Frasol®) in 0.2 ml *normal saline was injected subcutaneously in* the back—a total of 12 injections.

Group II

15 mice were casein treated as described above. In addition 12 subcutaneous injections of 0.2 ml normal saline were given as described for nitrogen mustard above.

Group III

12 mice were treated essentially as those of group II. At the end of 4 weeks a course of 3 nitrogen *mustard injections was administered over* 5 days (each dose 0.02 mg/0.2 ml normal saline).

Group IV

16 mice were treated for 4 weeks with daily subcutaneous injections 5 times a week of 0.5 ml normal saline—a total of 20 injections. Simultaneously nitrogen mustard was administered as described for group I.

The day after the last injections all animals were killed with ether inhalation and the following tissues were fixed in neutral formalin: spleen, liver, kidney, adrenal, ilium, lymph node, lung and thymus. Paraffin embedded sections were stained with methyl green pyronin, haematoxylin eosin, alkaline Congo red and the P.A.S. technique. The grading of amyloidosis in individual animals was a subjective estimation based on the broadness of the regular perifollicular amyloid rings in the spleens as described by *Christensen & Hjort* (1959).

TABLE 1

The Effect of Nitrogen Mustard on Amyloid Formation in Various Groups of Casein Treated and Control Mice

Group	Treatment	Number of mice developing amyloidosis/total	Mean degree of amyloidosis
I	casein + nitrogen mustard	11/15	++
II	casein + saline	15/15	++++
III	casein + saline *followed by* nitrogen mustard	*12/12*	+++++
IV	saline + nitrogen mustard	*1/16*	*0*

RESULTS

The main results of the present experiment are outlined in Table 1. It appears that both the incidence and severity of amyloidosis were re-

duced in casein treated mice simultaneously treated with nitrogen mustard (group I) as compared to mice casein treated alone (group II) In contrast mice which after the completion of 4 weeks of casein treatment received a short course of nitrogen mustard treatment (group III) showed an increase in the mean degree of amyloidosis as compared to mice casein treated alone That nitrogen mustard alone can not be considered amyloidogenic is evident from the figures of group IV

Among the animals of group I 4 mice failed to develop amyloid at all in spite of 4 weeks of casein treatment In these 4 mice the dominating histological feature was a marked depletion of lymphoid tissue especially pronounced in the spleen (Fig 1) which also showed a rather marked pyroninophilia P A S staining reticular cells were seldomly found In the remaining 11 mice splenic amyloid was found typically arranged around the depleted Malpighian corpuscles (Fig 2) in amounts ranging from grade + + to grade + + + (mean + +) Only a few of the animals of this group had recognizable amyloid deposits in liver or kidneys In contrast mice of group II which were casein treated for 4 weeks without receiving nitrogen mustard all exhibited a heavy splenic amyloidosis (Fig 3) ranging from grade + + + to + + + + + (mean + + + +) In addition all of these animals showed hepatic and renal amyloid deposits An even more pronounced amyloidosis was found among the animals of group III which *after* 4 weeks of casein received a short course of nitrogen mustard treatment In comparison only 1 out of 16 mice (group IV) nitrogen mustard treated for 4 weeks showed splenic amyloid and only to a very small extent The remaining 15 mice had no detectable signs of amyloidosis the predominant lesion being a rather heavy lymphoid depletion (Fig 4)

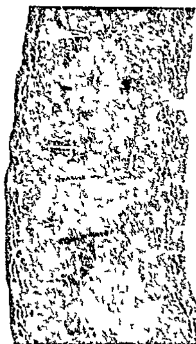
In group I pyroninophilia of splenic reticular cells was marked especially in the perifollicular areas In the groups II and III this pyroninophilia was found to be less pronounced while P A S positive reticular cells were frequently found in the border zones of the amyloid deposits In group IV pyroninophilia was modest

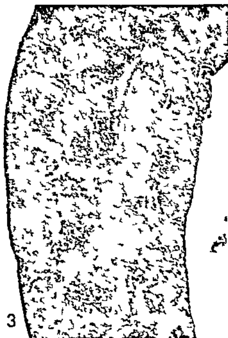
The amyloid was identified by its pink staining with Congo red which under crossed polars showed a brilliant green birefringence It was slightly eosinophilic and stained light purple with the P A S stain except for the small new formed amyloid deposits which stained vividly purple

DISCUSSION

The results of the present experiment indicate that nitrogen mustard when applied early during the course of an amyloidogenic treatment may inhibit and in some cases prevent amyloid formation Its mechanism of action in these respects is probably akin to the similar amyloidosis inhibiting effect of cortisone (*Christensen* 1961) and of antilymphocytic serum (*Ranløv* 1967) when applied from the beginning of the casein treatment In all these cases the effect most probably




2


3


4

relies on a suppression of the immune response which is believed to initiate the amyloidogenic process and which reflects itself in the pyroninophilia so characteristic of the reticular tissues (especially the spleen) during the initial preamyloid phase of experimental amyloidosis. Both in the experiments of *Christensen* (1961) using cortisone and in the present investigation a suppression or postponing of the primary pyroninophilic phase of amyloid formation was evident. However the present results show a somewhat less pronounced amyloidosis inhibiting effect of nitrogen mustard than that of cortisone and antilymphocytic serum. This is believed to be a question of dosage. If a sufficient suppression of the immune apparatus is not achieved the result may actually be an enhancement of the amyloidosis. This is illustrated by the acceleration of casein induced amyloidosis in mice subjected to adult thymectomy prior to the casein treatment (*Kanlov* 1966 a) or to sublethal irradiation (*Turunen & Teir* 1961).

The various experiments in which cortisone and nitrogen mustard have been shown to inhibit amyloid formation when applied *early* during the first few weeks of casein treatment viewed together with the experiments in which the same two compounds applied *late* in the course of an amyloidogenic treatment have been shown to enhance amyloid formation (*Teilum* 1952, 1954) are by no means conflicting. On the contrary they offer a perfect illustration of the two phase cellular theory of local amyloid formation (*Teilum* 1964). The initial preamyloid phase characterized by proliferation of pyroninophilic protein synthesizing cells is the presupposition of the second amyloid phase which is characterized by the functional break down of protein synthesizing cells in the face of a prolonged antigenic stimulation of the mesenchyme. The result is the deposition *in situ* of abnormal proteins (amyloid). *Inhibition of the former phase precludes the development of the latter.* Inhibition of the latter will promote the imminent break down thereby enhancing the subsequent amyloid formation.

Figs 1–4

Fig 1 Section of spleen from mouse after 4 weeks of combined treatment with casein and nitrogen mustard. The spleen shows marked lymphoid depletion but no amyloid. Haematoxylin eosin × 35.

Fig 2 Section of spleen from mouse after 4 weeks of combined treatment with casein and nitrogen mustard. The spleen shows lymphoid depletion and small amounts of amyloid arranged in the perifollicular regions (Grade ++). Haematoxylin eosin × 35.

Fig 3 Section of spleen from mouse after 4 weeks of casein treatment showing grade ++++ amyloidosis. Haematoxylin eosin × 3

Fig 4 Section of spleen from mouse after 4 weeks treatment with nitrogen mustard. Severe lymphoid depletion, no amyloid. Haematoxylin eosin × 3

SUMMARY

It is known that cortisone may inhibit amyloid formation in mice when administered prior to and during the first few weeks of casein treatment while it may enhance the amyloidosis when given late in the course of an amyloidogenic treatment A similar amyloid enhancing effect of nitrogen mustard has been described

In order to evaluate the effect of the time of administration nitrogen mustard was injected into groups of mice at various stages during casein treatment It appeared that both the the incidence and severity of amyloidosis was reduced in casein treated mice simultaneously treated with nitrogen mustard from the beginning of the experiment as compared to mice casein treated alone

The results are discussed in light of the two phase cellular theory of local amyloid secretion

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THE FUNCTION OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

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A spontaneously occurring lipid depletion of the adrenal cortex in mice of the AKR/O strain has been described by *Arnesen* (1955 and 1956) The lipid depletion coincides with sexual maturation in both sexes and is due to the effect of a single recessive gene the adrenocortical lipid depletion gene Prepubertal gonadectomy prevents the lipid depletion from taking place Hybridization experiments were carried out between the high leukemia AKR/O and the non leukemic WLO strain One of the resulting hybrid lines designated CS has maintained the adreno cortical lipid depletion and the inherited susceptibility to lymphatic leukemia derived from AKR/O strain however with a slightly reduced incidence of leukemia (*Arnesen* 1963 and 1964 a) The lipid content of the adrenal cortex can easily be estimated by inspection of the glands through laparotomy during life or by autopsy The normal lipid rich adrenal is bright yellow whereas the lipid depleted adrenal is red The origin and characteristics of the WLO strain which has been used for comparative studies in the present work are described by *Kreyberg* (1952)

It is not possible to make any generalization as to a correlation be tween the amount of lipid as seen histologically in frozen sections stained with Sudan III and the functional state of the gland An adrenocortical lipid depletion as such is compatible both with hyper function and with hypofunction of the adrenal cortex *Metcalf* (1960) who confirmed the existence of the spontaneous adrenocortical lipid depletion described by *Arnesen* (1956) in the AKR strain stated that the adrenal in AKR mice is hypofunctional with respect to production

Fellow of the Norwegian Cancer Society (Landsforeningen mot Kreft)
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Fig 1
CS male 12 weeks old *Adrenal gland* Spontaneous cortical lipid completion
Sudan III × 43



Fig 2
WIO male 12 weeks old Adrenal gland Normal cortical lipid pattern
Sudan III × 43

of lymphocytolytic steroids *Levine & Treiman* (1964) found a decreased response to stress in AKR mice The authors observed an impaired rise of plasma corticosterone levels in AKR mice after an electric shock when compared with control animals *Arnesen* (1964 b) on the other hand found that in mice with spontaneous adrenocortical lipid depletion treatment with cortisone reversed the lipid pattern and the cytological details of the cortical (*fasciculata*) cells to conditions found in most strains of mice These observations suggested that the spontaneous lipid depletion might be the result of increased pituitary stimulation of the adrenal cortex The author stressed however that definite conclusions in this respect were not yet possible

The purpose of the present study is to investigate the adrenocortical function in male CS mice and in males of the WIO strain In Figs 1

and 2 the characteristic lipid pattern of the two strains are depicted Fig 2 representing the normal one We were primarily interested in measuring the plasma corticosterone levels in ACTH stimulated animals Furthermore it was aimed to determine the corticosterone content of the adrenals in resting animals and to carry out *in vitro* studies on the production of corticosteroids in adrenal glands incubated with ACTH

MATERIAL AND METHODS

Animals

Male mice of the CS and WLO strains were used In order to procure CS mice for the experiments all matings of all animals since 1963 have been arranged by the author in the Department of Pathology Ullevål Hospital The strain has been maintained by brother-to sister matings Since 1962 in the same institution the author has arranged all matings of WLO animals to raise a sufficient number of WLO males for the present study The strain has been maintained by brother-to sister matings or father-to daughter matings if a brother was lacking

As a routine the young mice were separated from their parents and littermates at weaning during their 4th week The males were kept isolated in single cages measuring 12 × 29 × 12 cm with opaque plastic walls to their 12th week at which time they were killed When plasma corticosterone was measured in 3-week-old prepubertal mice the animals were not removed from the breeding cages until the day of experiment However when the study included investigations of adrenals from prepubertal male the animals were isolated from the age of 3 weeks The mice were then kept in single cages as outlined above for another week before the experiments were carried out

All details concerning the conditions in the animal house the housing and feeding of the mice are given elsewhere (*Solem* 1966) The mean weights of the animals are given with the results (see the tables)

All possible precautions were taken that CS mice and controls were raised and housed under identical conditions During the variou procedures included in the investigations the animals were handled in the same wa and great care was taken that every step of each experiment should be identical for the CS and WLO mice In all experiments where blood or adrenal tissue was collected from CS mice it was confirmed by inspection that the gross colour of the adrenal glands corresponded to the age of the animals In 12 week-old CS males the lipid depleted adrenals have a deep reddish colour resembling the colour of kidney tissue without a trace of yellow as previously described by *Arnesen* (1956) in the AKR/O mouse Plasma and adrenals from young CS males 3–4 weeks of age were only included in the present study when gross inspection had shown that the adrenals had the usual bright yellow colour found in most normal strain of mice indicating that lipid depletion had not yet taken place As a rare find ng the colour of the adrenal glands in these young CS males was appraised to be doubtful or even reddish and such animals were excluded from the experiments

Experimental Procedures and Agents

According to our previous experience it is extremely difficult to obtain reliable true re ting values of plasma corticosterone in mice (*Solem* 1966) In the present study we therefore carried out a very limited number only of plasma analyses in resting CS and WLO mice 12 weeks of age The data not included in th s paper showed no significant strain difference in the plasma cort costeroids of adult males However in several experiments pla ma c rtico te one was measured in blood collected from animals stimul ted with ACTH As a standa d procedure 100 mU were given subcutaneously in a single inje ti n and the animals sacrificed 60 minutes thereafter All details of the perform n e of the ACTH stimulat on are d scussed in a pre ious paper (*Solem* 1966 M e of the two t ains were always injected w th the same b tch number of c rt tr ph n[1]

[1] The following bat h number of A ortan (Acto ortin) Ferring A B Malmo Sweden were used 63031 and 6501?

We were interested in examining a possible difference in the corticoid content of the lipid depleted adrenal glands of CS mice and the normal adrenals of WLO mice The material for these studies was provided from 12-week-old resting males of the two strains

The out put of corticosteroids from the adrenals was investigated by *in vitro* studies Adrenal glands from resting CS mice and controls were incubated with ACTH peptide and the amount of corticoids was measured in the incubation medium The adrenals from the two strains were always incubated with the same batch number of ACTH peptide[2]

Measurement of Plasma Corticosterone

The sampling of blood was performed by collecting arterio venous blood in heparinized centrifuge tubes from the trunk portion of mice rapidly decapitated with a pair of scissors without anaesthe ia When blood was pooled the lipid con tent of the adrenals of each mouse was always estimated through inspection of the colour of the adrenals *post mortem*

Determination of plasma corticosteroids was carried out on 0 50 ml aliquots of plasma according to a modification of the fluoremetric method of *Silber Busch & Oslapas* (1958) and *Guillemin Clayton Smith & Lipscomb* (1958) elaborated and evaluated by *Solem & Brinck-Johnsen* (1965) The levels of corticoids were ex pressed as µg corticosterone per 100 ml plasma The 0 50 ml plasma aliquot for analysis derived from blood pooled from 2 to 3 mice Occasionally an aliquot of 0 25 ml plasma was analysed

In a limited number of 1 0 ml plasma aliquots we separated the fractions of bound and unbound corticosteroid using gel filtration The corticosteroids in the different fractions of the eluate were determined as corticosterone with the fluoro-metric procedure referred to above The gel filtrations were carried out according to the method described by *De Moor et al* (1962) and the details of the performance of the gel separation in our laboratory are presented in a preceding paper (*Solem* 1966)

Measurement of Adrenal Corticosterone Content

All precautions were taken to obtain the adrenal glands while the animals were in a resting state at 08 00 a m A procedure identical to that described in a r cent study (*Solem* 1966) to attain true resting values of plasma corticosterone was used The animals were killed by cervical dislocation CS and WLO males were sacrificed alternatingly The adrenals were rapidly removed immediately frozen on dry ice and kept frozen for 24 hours The glands were then carefully dis ected at room temperature The adrenals from 10 animals of each strain were separately pooled weighed on an analytical balance and thoroughly disintegrated in 10 ml distilled water using a MSE Homogeniser[3] To the tissue suspensi n wa added another 10 ml distilled water which simultaneously was used for removing particles of adrenal tissue adherent to the cutter blades and washing them into the glass container The 20 ml diluted tissue suspension in the container was then divided as exactly as possible in tw equal parts and transferred to two glass stoppered cen trifuge tubes and from this step on the analysis was carried out in duplicate The procedure was the following

The approximately 10 ml tissue suspension in each tube was extracted thrice with 15 ml methylene chloride as described in a previous paper (*Solem & Brinck-Johnsen* 1965) The total extract was gradually evaporated under N and ultimately brought to dryness The residue was dissolved in 2 ml petroleum ether by gentle inversion of the tube by hand 10 times A partition with 70 per cent ethanol was performed first with 10 ml once then twice with 6 ml Between each partiti n which was performed by shaking 100 times by hand the first time gently and later more vigourously a 5 minutes centrifugati n at 2 000 r p m[4] was carried out The ethanol

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[2] ACTH peptide Ferring AB Malm Sweden Batch nr 124 140 IU (intra venously) per mg

[3] Measuring and Scientific Equipment Ltd L ndon England (Catalogue No 7700) Propeller type

[4] g value 1080

was evaporated under N to a volume of about 15 ml and extracted thrice with 15 ml methylene chloride The final extract was evaporated under N and brought to dryness and was ready for chromatography The chromatography was carried out as previously described (*Solem & Brinck Johnsen* 1965) For computing purposes duplicates of a corticosterone standard 0 30 µg with 10 ml distilled water added and a water blank of 10 ml were carried through the whole procedure along with the adrenal tissue samples To locate the corticosterone derived from the adrenal tissue suspension 24 µg corti osterone were simultaneously chromatographed on a vacant strip of the same paper The further procedure was then as described pre viously when carrying out measurements of plasma corticosterone after chromato graphy (*Solem & Brinck-Johnsen* 1965) The amount of corti osteroids was ex pressed as µg corticosterone per 100 mg adrenal tissue and per pair adrenal glands

A description of the reagents and equipment used in these procedures was pre sented in the paper by *Solem & Brinck-Johnsen* (1965)

Measurement of the Corticosterone Production by Mouse Adrenals Incubated with ACTH

The *in vitro* method described here i almost the same as that of *Saffran & Shally* (1955) as modified by *Van der Vies* (1957) Sixteen mice (8 CS and 8 WLO males) were used for each experiment All precautions were taken to avoid exciting the animals and causing a possible activation of the pituitary adrenocortical system on the day of exper ment which started at 08 00 a m The singly housed animals were killed one by one by rapid cervical dislocation CS and WLO mice were sacri ficed alternatingly The adrenals were remo ed from each mouse rolled gently on a dry filter paper freed from adhering tissue carefully bisected and each half was placed on one of two pieces of tinfoil kept in a petri dish lined with moistened filter paper The bisection was performed as accurate as possible so that two almost equal halves were cut from each adrenal The two petri dishes one for the adrenal hal es from the CS mi e distributed on two tinfoil pieces and another correspondingly con taining the WLO adrenal halves were kept cooled on crushed ice At the end of distribution the four tinfoil pieces with the adrenal tissues were weighed on an analytical balance The adrenal halves were then placed in four 50 ml glass stop pered Erlenmeyer flasks Each flask contained 5 ml of Krebs Ringer bicarbonate glucose medium prepared according to *Van der Vies* (1957) and whi h had pre viously been bubbled with a mixture of 95 per cent O and 5 per cent CO_2 for 10 minutes The 4 flasks were mounted well flushed with the gas mentioned above and shaken at 38 C for one hour (prein ubation period) After the preincubation period the medium from the flasks was sucked off as completely as possible and replaced with 5 ml of fresh medium To each flask was then added 3 5 IU ACTH in a volume of 0 1 ml and the flask again well flushed with the mixture of 95 per cent O − 5 per cent CO The adrenals were then shaken for two hours (incubation period) At the end of the first hour the same dose of ACTH was added to the medium making the t tal amount of ACTH in each flask 7 IU After the incubation period the medium was removed to four glass stoppered centrifuge tubes labelled CS I CS II WLO I and WLO II For the transfer 5 ml distilled water was used for each flask to secure the complete removal of the med um From this step the analysis was carr ed out in duplicate according to the following procedure

The med um was extracted twice w th 15 ml methylene chlo de as d scribed in a preceding paper (*Solem & Brinck Johnsen* 1965) The total extr ct was gradually evaporated under N and finally brought to dryne and was then re dy for chromatography The chromatography was carr ed out in the ame way as described earlier (*Solem & B inck-Johnsen* 1965) Duplicate of a orticosteron standard 1 2 µg with 10 ml destill d water added and a water blank of 10 ml we e run with the extracts of the incubation media To one of the t ps of chromatography paper were applied 24 µg corticosterone for locat ng th orticosterone de i ed f om the extracts and the standard Concerning th f n l p o edu e ncluding elut on and fluorometric measur ment of the cortico te i we r fer to the above menti ned publication The amount of corticoste oid m ur d n the medium after 2 hours

[5] The ACTH pept le v as d ssolved in 0 075 N a etic acid and diluted with sal ne to a concentration f 35 IU per ml The solut on w s freshly prepared f r each experiment

incubation of the adrenals with ACTH was expressed as µg corticosterone per 100 mg adrenal tissue and per two adrenal glands

Statistical Methods

Differences were tested for statistical significance by means of the t-test. Precision has been assessed from pairs of measurements by means of the standard error of measurement

$$\sqrt{\frac{\sum D}{2n}}$$

where D is the differences and n the number of pairs

RESULTS

Plasma Corticosterone Levels after Stimulation with ACTH

Sixty minutes after subcutaneous injection of ACTH the plasma corticosterone in 12 week old male mice of the CS strain had reached a level which was distinctly lower than that observed in stimulated control animals (Table 1). The *response to ACTH in 3 week old CS* males was on the other hand virtually identical with that obtained in WLO male mice of the same age.

TABLE 1

Plasma Corticosterone in 3- and 12-Week-Old CS and WLO Male Mice 60 Minutes after Subcutaneous Injection of 100 mU ACTH

Animals	Age (weeks)	No. of samples (each a pool from 2 to 3 mice)	Plasma corticosterone (µg per 100 ml) Mean	Range	Standard deviation
CS	3	10	51.7	44.9–62.3	3.6
WLO	*3*	*10*	*53.0*	*45.1–64.8*	*3.6*
CS	12	20	32.2	27.1–37.2	3.2
WLO	12	20	42.1	34.4–47.0	4.2

Mean weight
CS mice 3 weeks old 12.2 g (range 8.5–14.5 g)
CS mice 12 weeks old 23.9 g (range 19.5–25.5 g)
WLO mice 3 weeks old 11.3 g (range 8.0–13.0 g)
WLO *mice 12 weeks old* 22.1 *g (range 16.5–24.0 g)*

Using gel filtration we separated the fractions of bound and unbound corticosteroids in plasma pooled from 12 week old animals after ACTH stimulation. The animals had been housed isolated from weaning. Table 2 shows that the reduced response to ACTH observed in adult CS males is due to a repressed increase of both fractions of corticosterone.

Adrenal Corticosterone Content

The content of corticosterone measured in the adrenal glands in resting CS males 12 weeks of age was found to be lower than that of the controls (Table 3).

TABLE 2

Plasma Corticosterone in 12-week-old CS and WLO Male Mice 60 Minutes after Subcutaneous Injection of 100 mU ACTH The Corticosterone Was Measured before (Regular Procedure) and after Gel Filtration All Data Represent Means of Duplicate Samples

Experiment No		Regular procedure Plasma corticosterone (µg per 100 ml)	Gel filtration Plasma corticosterone (µg per 100 ml) Unbound	+ bound	= total	Recovery %
CS mice	1	31 4	15 8	14 3	30 1	95 9
	2	33 0	17 1	15 1	32 2	97 6
Mean		32 2	16 5	14 7	31 2	96 8
Standard deviation		1 1			1 5	
Standard error of measurement (based on the differences observed in the duplicate)		0 6	0 7	0 6		
WLO mice	1	42 0	22 8	17 3	40 1	95 5
	2	43 1	24 4	17 5	41 9	97 2
Mean		42 6	23 6	17 4	41 0	96 4
Standard deviation		0 8			1 3	
Standard error of measurement (based on the differences observed in the duplicates)		0 6	1 1	1 3		

For each experiment was used a plasma pool obtained from 10 to 15 mice

TABLE 3

Corticosterone Content in Mouse Adrenals. The Animals Were 12-Week-Old CS and WLO Males Singly Housed and Resting. All Data Given Represent the Means of Duplicate Samples

Experiment No.	No. of mice	Corticosterone content µg/100 mg adrenal tissue	Corticosterone content µg/pair adrenals	Weight of adrenal pair mg
CS mice				
1	10	0.502	0.0106	2.11
2	10	0.417	0.0101	2.42
3	10	0.412	0.0091	2.21
4	10	0.398	0.0092	2.31
Mean		0.432	0.0098	2.26
Standard deviation		0.047	0.0007	
Standard error of measurement (based on the differences observed in the duplicates)		0.03	0.0007	
WLO mice†				
1	10	0.597	0.0120	2.01
2	10	0.606	0.0143	2.36
3	10	0.644	0.0139	2.16
4	10	0.555	0.0122	2.20
Mean		0.600	0.0131	2.18
Standard deviation		0.037	0.0012	
Standard error of measurement (based on the differences observed in the duplicates)		0.02	0.0005	

Mean weight CS mice 24.1 g (range 20.5–26.5 g)
† Mean weight WLO mice 22.3 g (range 17.5–25.0 g)

The Corticosterone Production by Mouse Adrenals Incubated with ACTH

Table 4 shows the results of the *in vitro* studies. The production of corticosterone by adrenal glands of 12-week-old CS males was found to be markedly lower than by the adrenals from WLO male mice of the same age. However, the corticosteroid production in adrenals from prepubertal males of both strains was observed to be within the same range (Table 5).

DISCUSSION

The lipid depletion of the adrenal cortex in mice of the CS strain is known to coincide with sexual maturation.

The observations made in the present study have not revealed any difference in the adrenocortical function of prepubertal CS males, 3 to 4 weeks old, and of WLO males of the same age.

TABLE 4

Corticosterone Production in vitro in Mouse Adrenals Incubated with ACTH The Animals Were 12-Week-Old CS and WLO Males Singly Housed and Resting The Adrenals Were Removed and Bisected After Preincubation the Adrenal Halves Were Incubated with ACTH for 2 Hours and the Amount of Corticosterone in the Medium Was Measured All Data Given Represent the Means of Duplicate Samples

Experiment No	No of mice	Corticosterone production µg/ 100 mg adrenal tissue/2 hours	Corticosterone production µg/ 2 adrenals/ 2 hours	Weight of 2 adrenals mg
CS mice				
1	8	44 19	1 21	2 73
2	8	43 03	1 10	2 55
3	8	43 14	1.26	2 93
4	8	46 93	1 41	3 01
5	8	44 35	1 13	2 54
6	8	39 62	1 03	2 60
Mean		43 54	1 19	2 73
Standard deviation		2 38	0 13	
Standard error of measurement (based on the differences observed in the duplicates) *		2 25	0 07	
WLO mice†				
1	8	88 66	2 31	2 61
2	8	83 36	2 41	2 89
3	8	83 69	1 84	2 21
4	8	86 13	2 05	2 38
5	8	88 92	2 29	2 57
6	8	88 19	2 26	2 55
Mean		86 49	2 19	2 54
Standard deviation		2 54	0 21	
Standard error of measurement (based on the differences observed in the duplicates)		6 04	0 13	

* Mean weight CS mice 24 6 g (range 20 0–26 0 g)
† Mean weight WLO mice 22 9 g (range 17 0–26 0 g)

The results of the investigations performed on adult 12 week old male mice are on the other hand pointing towards a reduced production of corticosterone by the adrenal glands of CS males of this age

First this assumption is supported by the results of the *in vitro* studies

Second, the poorer response to corticotrophin measured by the repressed increase in plasma corticosterone levels after ACTH stimulation suggests hypofunctional adrenals in adult male mice Since the peripheral plasma corticosteroid levels represent a balance between the rates of synthesis release utilization and degradation of the hormone it is more difficult to interpret the results of plasma analyses

TABLE 5

Corticosterone Production in vitro in Mouse Adrenals Incubated with ACTH The Animals Were 4-Week-Old CS and WLO Males Singly Housed and Resting The Adrenals Were Removed and Bisected After Preincubation the Adrenal Halves Were Incubated with ACTH for 2 Hours and the Amount of Corticosterone in the Medium Was Measured All Data Given Represent the Means of Duplicate Samples

Experiment No	No of mice	Corticosterone production µg/ 100 mg adrenal tissue/2 hours	Corticosterone production µg/ 2 adrenals/ 2 hours	Weight of 2 adrenals mg
CS mice*				
1	8	96 88	1 24	1 28
2	8	91 49	1 18	1 29
Mean		94 19	1 21	1 29
Standard deviation		3 81	0 04	
Standard error of measurement (based on the differences observed in the duplicates)		3 05	0 29	
WLO mice†				
1	8	92 11	1 16	1 26
2	8	87 90	1 12	1 30
Mean		90 01	1 14	1 28
Standard deviation		2 82	0 03	
Standard error of measurement (based on the differences observed in the duplicates)		4 61	0 10	

* Mean weight CS mice 12 5 g (range 9 0 14 5 g)
† Mean weight WLO mice 11 6 g (range 8 0 13 0 g)

However held together with the outcome of the incubations of adrenals with ACTH it seems justified to conclude that the results of the plasma analyses too are speaking in favour of an adrenal hypofunction

Third the lower corticosterone content demonstrated in the adrenal glands of 12 week old CS males is consistent with a hypofunction or at least is a finding which is not contradictory to a decreased *production* of corticosterone by the adrenals However no generalization is possible as to the relationship between the corticosteroid content and the secretory rate of the adrenal glands Our finding gives on the other hand additional support to the statement of *Arnesen* (1963) that the storage of steroids is reduced or abolished in permanently depleted adrenal glands

The adrenal cortex of adult 12 week old male mice of the CS strain is known to be lipid depleted The adrenocortical lipid depletion as *such says nothing as to the functional state of the CS adrenal cortex* Loss of lipids of the *adrenal cortex might as well be found after hypo*physectomy as be the result of hyperfunction of the gland in the

latter case indicating a discharge of cortical lipids Taken one by one none of the findings in the present study is necessarily indicative of adrenal hypofunction However their association makes it likely that the adrenal gland of the adult male CS mouse is hypofunctional at least with respect to corticosterone production

The last reservation is taken with an eye to the finding of *Arnesen* (1963) that the cortical cells in the lipid depleted adrenals of the adult CS mice were very rich in mitochondria Arnesen stated that this finding was hardly compatible with the concept that the spontaneous lipid depletion reflected a state of permanent hypofunction of the cortex as postulated by *Metcalf* (1960) Arnesen stressed however that the term function in this connection was equivocal One thing was the total or partial function in terms of specific hormone production another was the basic and unspecific metabolic activity of the cells The statement of Arnesen and the findings in our present study are therefore not necessarily conflicting The possibility remains that the synthesis of corticosterone in the adrenal glands of the adult CS male in some way or other is reduced In order to secrete the corticosteroids necessary to assure homeostasis the basic metabolic activity of the cortical cells might be enhanced and the cells present themselves as hyperactive in the ultrastructural picture as shown by *Volbert & Arnesen* (1960) More definite conclusions concerning the hypothesis here advanced are not yet possible

SUMMARY

The adrenocortical function of male mice of the CS strain has been investigated The prepubertal CS mice are known to have normal lipid pattern of the adrenal cortex whereas the elder adult CS mice have a spontaneous permanent lipid depletion of the adrenal cortex The experiments included measurements of plasma corticosterone in ACTH stimulated animals determinations of the corticosterone content of the adrenals in resting mice and measurement of the corticosterone production *in vitro* in adrenals incubated with ACTH The inbred strain WLO with normal adrenocortical lipid pattern was used for comparative studies

The study did not reveal any abnormalities in the adrenocortical function of prepubertal CS male mice The result of the investigations on adult CS males 12 weeks of age were highly suggestive of reduced production of corticosterone by the adrenal cortex

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CHROMOSOMAL ANALYSES OF ROUS SARCOMAS IN MICE—COMPARISON BETWEEN THE FINDINGS IN THE TUMOUR AND IN MATERIAL EXPLANTED IN VITRO

By

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In a previous investigation the chromosomal picture was analysed in 91 primary Rous sarcomas in mice (*Mark* 1967 b) About 40 per cent of the tumours had a normal diploid stemline Among the heteroploid sarcomas the stemline numbers ranged from 37 to 84 Hyperdiploid and tetraploid tumours 28 and 14 per cent respectively were the other two major components of the chromosomal spectrum while hypodiploid pseudodiploid and triploid sarcomas formed minor groups These results were based on the findings obtained in fixations taken directly from the tumour (D) In fixations from tumour material explanted *in vitro* for a short period (Pc) 20 hours–7days a deviating chromosomal picture was a common finding A comparison between the findings in D and Pc from the same sarcoma was possible in the 81 tumours studied by both methods

Among the 81 sarcomas 49 had a heteroploid stemline in D In Pc only about 1/ of these heteroploid sarcomas showed the same or a similar stemline however and nearly 3/4 had a normal diploid stemline In this last group the stemline found in D had disappeared completely in approximately /3 of the cases and in approximately 1/3 it was refound as a sideline Finally a small group about 8 per cent of the sarcomas with a heteroploid stemline in D showed a new heteroploid stemline in Pc This *in vitro* normalization of the chromosomal picture naturally did not apply to primary sarcomas with normal diploid stemline in D Many of them however had one or several heteroploid sidelines which were sometimes subjected to normalization *in vitro* As in previous group there were only a few instances of new heteroploid stem sidelines in Pc

This type of difference between D and Pc manifested as occurrence

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TABLI 1

Stemline in vivo (D) and in vitro (Pc) in 2f Primary Tumours Subjected to Cross Tests

Tumour no	Sex	Stemline in D marker(s) — +	The stemline found in D is in Pc: the same	a sideline Stemline has normal diploid karyotype	not found Stemline has normal diploid karyotype	Results of cross tests in table
2	♀	37	−	−	+	2
3 sample I	♂	38				
5 sample I	♀	39	−	−	+	
12	♂	40	(+)	−	−	3
15	♂	40	(+)	−	−	
18	♂	40	(+)	−	−	
33 sample I	♂	40	(+)	−	−	
38 sample I	♀	40	(+)		−	
39 sample II	♀	40	(+)	−		
40	♀	40	(+)	−	−	
45	♂	40+	−	−	+	4
4f	♀	40+	+	−	−	
49	♂	41	+	−	−	5
54	♀	41	−	−	+	
5f	♀	41	−	+	−	
33 sample II	♂	4?	−	−	+	
57 sample III	♂	42	−	−	+	
f4	♀	42	−	−	+	
65	♂	42	−	−	+	
f7	♂	42	+	−	−	
70	♂	43	−	+	−	
75	♂	5?+	−	+	−	6
6	♂	57	−	−	+	
36 samp le II	♀	67	−	−	+	
81	♂	74+	−	−	+	7
89	♂	80	−	+	−	

Only investigated in Pc but in op ration ample II IV the same picture *n vitro* as for tumour no 2 and no

of new heteroploid stem sidelines in Pc was regarded as an example of differential growth rate *in vitro* for tumour cells as discussed earlier (*Mark* 1967 b) Concerning the other type of difference between D and Pc apparent as a normalization of the chromosomal picture in Pc arose the question of the real nature of the cells with a normal diploid karyotype Two possibilities seemed to exist (1) Outgrowth *in vitro* of normal stroma cells present in the cultured tumour fragments (2) Overgrowth *in vitro* of a common second tumour stemline with a normal karyotype The scope of the present investigation is to solve this problem It might be performed by transplanting a tumour to a host with a sex opposite to the host for the primary tumour and then determining the sex *in vitro* of the outgrowing cells from explant of the transplanted tumour

MATERIAL AND METHODS

The material consisted of 24 different primary Rous sarcomas in mice The sex of the host and the designation for each primary tumour is given in Table 1 The number ng is the same as in previous investigation (*Mark* 1967 b) In those cases where a primary tumour has been studied se eral times using specimens from subtotal operations that utilized for the present experiment is indicated by the sample number Table 1 also shows the stemline numbers for each primary tumour and the occurrence of marker(s) The following columns illustrate the relation ship between the findings in D and Pc of the primary tumours Among primary sarcomas with heteroploid *stemline the material includes three* with similar or the same pi ture in D and Pc four with a partial normalization of the chromosome picture *in vitro* and 12 with a complete normalization The number in the last column refers to the table with the results for each tumour group

The primary tumours were induced in mice of the inbred strain CBA by inocula tion of a finely minced suspension of Rous chicken sarcoma strain Schmidt Ruppin (*Mark* 1967 b) All animals used for transplantation were adult mice of the same strain The primary tumours were transplanted during 1–11 passages The new *hosts were consistently selected to be of opposite sex to that of the mouse in which* the sarcoma was induced (cross test)

The changes of the stemline of the primary tumour during transplantation were followed by direct fixations (D) The findings in direct fixation from the trans planted tumour served as a control of those from the primary culture (Pc) When a sarcoma was transplanted for several generations both D and Pc were performed intermittently

All transplanted tumours were studied in Pc after 2 10 da s of growth *in vitro* *In addition most sarcomas were investigated in Pc after a ery short t me of* cultivation about 10–36 hours and after a comparatively l ng time *n v tro* 11–29 days Often these P s were performed as a consecutive study of one o seve al passages

The meth ds used for D and Pc were described earl er (*Mark* 1967 a) All of the 199 determinations (Pcs) of stromal percentage ha e been made on at least 50 cells in each case except 10 in which the number of ell we e on an ave age 33 and at least 25 The ex was determined karyot pe nal ses were made by photo *graphing some 4 5 cells in each tumour steml ne and oft n al o of each sid l ne*

As in previous reports stemline S is used to den te the m st frequent karyotype in the populations of tumour cells and sid l ne ((3) s used to denote other karyotypes o curring n a frequen f about 10 per cent or more of the population

OBSERVATIONS

Diploid and hyperdiploid primary tumours were the two categories studied most extensively but all stemline groups which appeared in

the material of primary tumours were represented in the present investigation (Table 1) The results for the different tumour groups are recorded in Tables 2–7 which show the duration of the primary culture and the percentage of stroma cells appearing in each Pc The stemline among tumour cells in vitro and the stemline in D respectively are found in the two last columns

The cross tests i e transplantations of male or female tumours to hosts of opposite sex followed by sex determination of the cells in Pcs revealed the same fact in all cases Metaphases with a normal

TABLE 2

Cross Tested Tumours with a Hypodiploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	
2	I	36h	94	–	37
		5	98	–	
	II	20h	8	37	
		5	96	–	
	III	20h	0	37	37
		4	70	37	
		5	98	–	
	IV	20h	10	37	74
		5	100	–	
	VII	20h	10	36	64+
		4	92	–	
		10	94	–	
	VIII	20h	6	60–61+	60+
	X	24h	6	58+	58+
		5	52	57+	
	XI	7	78	57+	57+
		15	90	–	
3 sample I	I	6	100	–	
	II	4	78	38	38
	III	5	96	–	
		16	66	38	
	IV	20h	0	74	74
		5	92	–	
	V	12h	14	76	
		24h	78	76	
		5	94	–	
		23	82	39	
	VII	20h	4	74	74
		6	80	38	
		14	62	77	
5 sample I	I	20h	2	39	39
		4	92	–	
		12	88	(39)	

no designation for days

TABLE 3

Cross Tested Tumours with a Normal Diploid Stemline

Primary tumour no	Passage no	Primary cultures: duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
12	I	24h	23	40	40
		5	80	40	
	III	11	80	40	40
	IV	3	33	40	
	V	4	48	40	
	VI	3	65	40	40
		10	94	–	
	VIII	24h	10	40+	40+
		2	46	40+	
				40	
	IX	20h	8	40	
		6	44	39–40	
	X	20h	4	40+	40+
		3	78	40+	
15	VII	4	56	40	40
	X	4	2	78	78
18	I	20h	25	40	40
		4	90	–	
		8	94	–	
	II	20h	14	40	40
		5	82	40	
		11	8	41	
	IV	20h	14	41	41
		2	68	41	
		4	82	41	
	V	5	40	41	41
	VII	36h	78	41+	41+
		5	82	41	
		15	100	–	
	X	24h	42	41+	41+
		4	94	–	
	XI	12h	2	41+	41+
		7	50	41+	
33 sample I	I	24h	48	41–42	41
		6	70	42	
38 sample I	I	20h	0	43	43
		4	96	–	
		12	84	40+	
38 sample II	I	20h	4	43	43
		4	86	–	
		15	45	43	
40	I	20h	6	40	40
		5	70	40	
		18	30	±80+	
	II	20h	0	80+	9+

TABLE 3 (cont)

Primary tumour no	Passage no	Primary cultures: duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
40		4	10	79+	
		11	10	79+	
	V	24h	6	77+	77+
		5	4	77+	
	VIII	20h	0	77+	77+
		7	2	77+	
	X	4	0	79+	79+
		10	2	80+	

TABLE 4

Cross Tested Tumours with a Pseudodiploid Stemline

Primary tumour no	Passage no	Primary cultures: duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
45	I	12h	9	40+	40+
		5	54	40+	
		12	98	–	
	II	12h	4	40+	40+
		24h	10	40+	
		7	90	–	
	V	20h	6	40+	40+
		9	98	–	
	X	10h	0	41+	41+
		6	4	41+	
		13	28	41+	
46	I	4	75	40+	40+
		7	92	–	
	IV	20h	4	40+	40+
				80+	
		4	18	40+	
	XI	12h	0	40+	40+
		3	3	40+	

diploid karyotype had the same sex as the host Thus they must represent stroma cells since this is the only component from the host present in the explants of the transplanted tumour Among the cross tested sarcomas with a normal diploid stemline most of the outgrowing cells were stroma cells (Table 3) too In all tumour groups however the degree of stromal growth was variable and above all influenced by the duration of the primary culture

TABLE 5

Cross Tested Tumour with a Hyperdiploid Stemline

Primary tumour no	Passage no	Primary cultures: duration days hours	Primary cultures: stroma cells per cent	Primary cultures: tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
49	I	3	9	41	41
		7	20	41	
		11	38	41	
	II	3	18	41	
		19	6	41	
	IV	20h	2	41	41
		4	0	82	
	VII	4	6	82	82
		19	0	43	
	X	2	28	40–41	40
		7	18	41	
		10	0	41	
54	I	20h	12	41	41
		7	98	–	
		18	88	(42)	
56	III	4	38	41	41
	VII	4	16	78	78
33 sample II	I	2	8	42	42
		4	74	41	
		14	66	42	
57 sample III	I	4	20	42	42
		13	20	42	
	III	4	98	–	
		7	96	–	
		17	92	42	
	IV	36h	98	–	42
		8	70	41	
	V	12h	8	81	81
		6	94	–	
		8	90	–	
	VI	2	64	79	79
		29	0	42	
	VIII	20h	28	81	81
		4	98	–	
		13	96	–	
	X	20h	12	74	81 74
		3	88	–	
		10	100	–	
64	I	20h	8	42	42
		7	94	–	
		11	96	–	
	II	4	26	41 42	42
65	I	20h	5	42	42
		4	96	–	
		8	92	–	

TABLE 5 (*cont.*)

Primary tumour no	Passage no	Primary cultures: duration days hours	Primary cultures: stroma cells per cent	Primary cultures: tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
65	II	5	98	–	
		12	98	–	
	IV	12h	16	43	43
		20h	26	43	
		5	96	–	
	VII	36h	98	–	43
67	I	3	2	43	42
	II	2	4	44	44
	IV	5	0	89	44
		7	23	44	
	V	3	4	87	87
	VI	3	48	85	85
	IX	3	20	42 84	42
		4	24	44	
	X	20h	2	42	42
70	I	20h	2	43	43
		6	96	–	
		10	98	–	

Before dealing with the details of this subject it might be convenient to survey the entire material in this respect. This is done in Table 8 and the mean values for the Pcs from all passages of all tumours are further plotted in the diagram in Fig. 1. In the entire material the percentage of stroma cells was lowest in fixations performed within 20 hours. During the following short period, 21–36 hours, the stromal values were increased to more than 50 per cent and then during the following 8 days there was a slow, gradual increase to values around 70 per cent. After 10 days, finally, the total averages indicated a fall in the stromal percentage.

10–20 hours. Already in the previous study of primary sarcomas (Mark 1967 a) it was found as a rule that fixations of Pcs performed before 24 hours after the explantation *in vitro* gave a result identical with or similar to the D. These observations were confirmed in the present study (Tables 2–8). The early Pc turned out to be an excellent method for chromosomal analysis of the Rous sarcomas and the results could be used for checking the findings in D. It is to be noted, however, that usually a minor fraction of stromal cells but occasionally a considerable fraction was found as early as 20 hours after cultivation (cf. passage I of tumour 18, passage I of tumour 40, passage VIII of tumour 57, sample III, and passage IV of tumour 65). A reduction of the duration of the primary culture to 10–12 hours usually caused



Fig 1

Mean percentage of stroma cells (in entire material 199 samples) after different periods of *in vitro* growth

an obvious decrease of the frequency of mitosing stromal cells in these cases (passage XI of tumour 18 passage V of tumour 57 sample III and passage IV of tumour 65) This indicates that normalization often proceeds rapidly It seemed to be a rule that the percentage of stroma cells was lower during the first 20 hours than during the following 9–10 days but there were exceptions with similar stromal values or

TABLE 6

Cross Tested Tumours with a Triploid Stemline

Primary tumour no	Passage no	Primary cultures: duration days hours	Primary cultures: stroma cells per cent	Primary cultures: tumour cells S and marker(s) = +	Stemline in D S and marker(s) = +
36	I	4	95	–	
sample II	III	2	92	–	62–63+
		3	94	–	
	IV	36h	65	62–63+	
		5	100	–	
	VII	12h	0	63+	63+
		5	98	–	
	X	20h	4	63+	63+
		3	58	62–63+	
		9	88	(61+)	
75	I	2	18	50+	50+
sample I		10	66	51+	
	II	20h	0	50+	50+
		4	8	50+	
		16	0	50+	
	VIII	5	18	49–50+	
	X	4	14	49–50+	49+
		8	6	49+	
		17	10	50+	
76	I	20h	24	54+	64+
		5	98	–	
		3	100	–	

TABLE 7

Cross Tested Tumours with a Tetraploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S a ? marker(s)
		duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	= +
81	I	4	30	74+	74−
	II	7	3	74−	
		16	94	−	
	IV	20h	0	74+	74−
		4	4	74+	
	VII	3	2	73−	73−
		13	4	73−	
		2	76	73−	
	X	3	12	70–71−	70–71−
		12	50	74+	
88	VI	20h	8	40, 78−	78−
		6	60	40	

even a decrease in the frequency during the latter period (e.g. passages VIII and X of tumour 40 and passage IV and X of tumour 49) which means that the tumour cells are sometimes capable of outgrowing the stroma from the beginning

21 hours 11 days The survey in Table 8 and the diagram in Figure 1 obscure the fact that there existed at least three different patterns of stromal overgrowth during this period. The first and most common pattern was characterized by a very high degree of stromal growth initially (often more than 90 per cent) and the high stromal [illegible] persisted during this period. Typical examples are found among the passages of tumour [illegible] sample III, tumour 65 and tumour [illegible]. A second pattern was characterized by a stromal overgrowth increasing with the duration of the primary culture. Passage I of tumour 45 and passage I of tumour [illegible] [illegible] stromal growth may proceed [illegible] different [illegible] I of tumour [illegible] it had reached a level of about [illegible] days, while this level was more than 90 per cent in passage [illegible] [illegible] approximately the same [illegible] [illegible] patterns were seen among primary cultures [illegible] [illegible] (Fig. 10 [illegible]). A third [illegible] uncommon [illegible] the period 21 hours [illegible] 0 days was a considerable variation in the [illegible] [illegible] and no [illegible] with the duration [illegible] [illegible] examples are found among passages of tumour [illegible]

[illegible] days During this period two patterns can be distinguished [illegible] stromal overgrowth. In the first a high degree of stromal [illegible] with persistent [illegible] from the period [illegible]

Typical examples are passage I of tumour 54 passage I of tumour 5 sample I and passage VI of tumour 2 The other more common pattern during this late period was characterized by a more or less evident decrease of stroma cells Examples are found among passages of tumour 57 sample III and passages of tumour 49 In these cases the decrease could be found in several passages but in other sarcomas it was only seen in one passage (f i tumour 18)

Summarizing the findings for the whole period of *in vitro* growth 10 hours–29 days the following four patterns can be distinguished among the passages of the 24 primary sarcomas

1 A continuous low fraction of stroma cells *in vitro* (example pas sages of tumours no 49 and no 75)

2 An initial low fraction of stroma cells gradually increasing and then usually decreasing in late Pcs (example passages of tumours no 3 and no 45)

3 An initial low fraction of stroma cells and a subsequent increase during the following periods but no definite correlation with the du ration of the primary culture (example passages of tumour no 12 and early passages of tumour no 57 sample III)

4 An initial often considerable fraction of stroma cells rapidly increasing to a high level persisting for a long time and only some times decreasing in late Pcs (example passages of tumours no 2 and no 65)

With regard to the four patterns mentioned above there were often striking similarities between different passages of the same tumour and between these and the findings in the primary sarcomas (Table 1) It indicates that the patterns were related to fairly stable and spe cific characteristics of the individual sarcoma and were not due to random influences However differences between passages of the same tumour were found in some cases A few instances of an abrupt change in the *in vitro* pattern were recorded (passage I versus passage III of tumour no 57 sample III passage I versus II of tumour 40 and passage V versus VI of tumour 67 are the most evident cases) In another cate gory there was no real change in the *in vitro* pattern only a more or less apparent decrease in the stromal overgrowth after the first day *in vitro* (passages of tumour no 46 and late passages of tumour no 36 sample II and no 45) In most instances the decrease was a parallel phenomenon to increase in passage numbers It is to be noted however that this was a tendency found in some cases and it was not a detect able trend for the entire material (Table 8)

No distinguishable differences could be found between different S number groups Neither was it possible to correlate the decrease in stromal overgrowth with increasing passage numbers with any special karyotypic change On the contrary the time patterns *in vitro* men tioned above often remained unchanged despite profound changes in the karyotype during transplantation This constancy was shown by

TABLE 8

Mean Percentages of Stromal Cells in Primary

Period of primary culture	S number of primary tumour						
	< 2x	2x normal karyo type	2x pseudo diploid karyo type	2x normal and pseudo diploid karyo type	> 2x	± 3x	± 4x
10–20 hours	6 0(9)	8 8(11)	3 8(6)	7 1(17)	11 0(11)	7 0(4)	4 0(2)
21–36 hours	59 3(3)	34 5(6)	10 0(1)	31 0(7)	98 0(2)	65 0(1)	–
2– 4 days	83 0(4)	56 9(15)	32 0(3)	52 8(18)	37 4(21)	54 1(7)	17 0(2)
5– 7 days	*89 5(11)*	*52 4(10)*	*60 0(4)*	*54 6(14)*	*61 3(12)*	*78 5(4)*	*19 8(4)*
8–10 days	94 0(1)	63 3(3)	98 0(1)	72 0(4)	74 7(6)	53 3(3)	–
11–16 days	76 5(4)	54 5(6)	63 0(2)	56 6(8)	69 0(6)	0 0(1)	49 3(3)
17–29 days	82 0(1)	30 0(1)	–	30 0(1)	23 2(5)	55 0(2)	36 0(1)

Figures in brackets = number of samples in each group

for example tumour no 2 no 3 and no 18 In the first case tumour no 2 the stemline was probably doubled during passages and several markers appeared but still no definite change could be found in the *in vitro* pattern However three cases with a probable correlation between karyotypic changes and changes in the *in vitro* pattern were found They appeared among the few tumours with an abrupt alteration of the *in vitro* pattern Thus in passage II of tumour no 40 there was a sudden decrease in stromal overgrowth at the same time as the stem line shifted to tetraploid level After the shift to tetraploidy the stem line has undergone minor changes except for the appearance of double minutes (*Mark* 1967 a) and the picture *in vitro* has been stable In passages of tumour no 49 and no 67 the hyperdiploid stemline was doubled but in late passages both were returned to the hyperdiploid region In connection with this reversal there was a definite increase in stromal overgrowth in both cases suggestive of a correlation with the karyotypic change

DISCUSSION

In the present investigation the same growth medium was used for all primary cultures *viz* Parker s tissue culture medium 199 supplemented with 20 per cent unfiltered inactivated serum from newborn calves Another factor which may be regarded as constant is the genotype of the stroma cells which were all CBA It is true that in different explants *the stroma cells may vary in relative frequency* and in their readiness for mitosis However their competitive potential *should be the same* Thus the cross tests actually constitute an estimate of the competitive capacity of the tumour cells versus the stroma cells Under the existing conditions of the *in vitro* milieu the tumour cells

tures after Different Periods of in vitro Growth

In vivo passage no											
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	Mean of entire material
5(11)	6 5(4)	0 0(2)	9 0(8)	9 3(3)	8 0(1)	4 7(3)	11 3(3)	8 0(1)	4 4(5)	1 0(2)	7 7(43)
(3)	10 0(1)	–	81 5(2)	4? 0(2)	–	88 0(?)	10 1(1)	–	24 0(2)	–	50 5(13)
(14)	33 0(4)	66 7(6)	34 ?(6)	26 0(?)	59 0(3)	4? 5(4)	72 0(2)	2? 0(?)	45.3(8)	3 0(1)	4? 7(52)
3(1?)	6? 5(6)	99 7(3)	68 3(6)	58 0(4)	60 0(1)	65 5(4)	10 0(2)	44 0(1)	21 5(4)	64 0(2)	63 9(45)
0(4)	–	–	70 0(1)	94 0(?)	94 0(1)	94 0(1)	–	–	39.2(5)	–	70 7(14)
9(8)	5? 5(4)	48 7(3)	–	–	–	55 3(3)	96 0(1)	–	39 0(2)	90 0(1)	60 0(2?)
(3)	6 0(1)	22 0(1)	–	8? 0(1)	0 0(1)	18 0(2)	–	–	10 0(1)	–	37 4(10)

were in general inferior With changed conditions however for ex ample different media diverging results are not excluded In the pre sent study only one type of environment was tested

The results obtained give a satisfactory explanation of the discrep ancies observed between D and Pc Moreover the results give an esti mate of the magnitude of an important source of error in this type of experiment which has so far often been neglected The common and often extensive stromal overgrowth *in vitro* was demonstrated for transplanted Rous sarcomas in mice However a partial or complete normalization was found in many primary Rous sarcomas subjected to consecutive Pcs there is no reason to believe that one was dealing with another factor in these cases

The generally good correlation between D and early Pcs performed *within 24* hours is probably not due to a real outgrowth of tumour cells It seems more plausible that tumour cells ready for mitosis or even in mitosis will complete the mitotic cycle probably after a short lag period due to disturbances at the explantation The critical period follows usually lasting for 10–14 days during which the stroma cells divide rapidly and outnumber the tumour cells The artificial conditions *in vitro* with exclusions of normal mechanisms for control of growth seem to favour the normal stroma cells at the expense of the tumour cells A shorter division cycle for the stroma cells is another factor which may influence the picture *in vitro*

The growth *in vitro* of normal cells in general starts with a period of rapid divisions followed by a period of stagnation and often degener ation The reappearance of a considerable number of tumour cells in old primary cultures is believed to be connected with this change in growth behavior of normal cells The final picture during long term

cultivation was not studied This would involve the question of transformation into permanent cell lines of either stroma cells tumour cells or both

Histologically it is not possible in most sarcomas to distinguish a *stroma cell from a tumour cell* Judging from the results of the present and previous analyses the stromal content of most murine Rous sarcomas is probably very high Rapid and extensive stromal overgrowth *in vitro* seemed to be especially common among sarcomas which were hard and fibrous and did not change their character during transplantation (typical examples are tumour no 3 sample I tumour no 57 sample III and tumour no 60) The differences between separate tumours were merely quantitative and the frequency of fibroblasts in an explant is probably of importance for the degree and rapidity of the normalization *in vitro*

Most sarcomas became softer during transplantation and the time for outgrowth of the tumour was usually reduced to 10–14 days A reduction of the stromal content parallelling the increased malignancy seems reasonable to assume Both factors are believed to be responsible for the tendency towards reduced stromal outgrowth *in vitro* in late passages of some tumours

Karyotypic changes of the stemline were not constantly connected with deviations in the *in vitro* pattern However the chromosomal progression is still in the beginning in most sarcomas studied above and probably much of the original characteristics of the tumour cells are unaltered Further a genotype successful *in vivo* is not necessarily successful in the *in vitro* milieu

No significant differences could be found in the *in vitro* pattern between separate classes of stemlines On the contrary each sarcoma seemed to follow its own pathway with regard to stromal overgrowth in primary cultures This tendency was strengthened by the striking similarities in the *in vitro* pattern between different passages of the same tumour

Despite the common use of *in vitro* methods for chromosomal analyses of tumours comparatively few investigations have been made of the relationship between D and Pc Methods like the cross tests have been used too seldom and generally it has been impossible to exclude tumour stemlines with a normal karyotype and superior *in vitro* to the heteroploid stemlines This controversial subject was discussed especially by *Sandberg et al* (1961 and 1962) dealing with acute leukaemias in man They found a significant increase of normal diploid cells as early as 10 hours after the initiation of the cultures and presumed that these normal elements represented nonmalignant cells They point out quoting experiences by *Baikie et al* and *Ford* that cultured leukaemic cells rarely grow as well as material from individuals without leukaemia and sometimes they do not grow at all *Baker & Atkin* (1965) studying human lymphosarcomas and Hodgkin's

disease also found a high frequency of normal cells in their primary cultures and in some of these cases figures are given which show an increase of the normal cells with the duration of the primary cultures

A similar situation seems to exist for bovine leucosis heteroploid stemlines in D often disappeared in Pc and normal cells took over Further there was occasionally a time pattern with a heteroploid stem line in the very early Pc and a normal finding in a later Pc (*Basrur et al* 1964 *Hare et al* 1964) Since stemlines with an apparently normal diploid karyotype have been found in D of both human and bovine leukaemia it cannot be concluded with certainty that the normal chromosome pictures in Pcs are due to overgrowth of normal cells but the suspicion is strong

Stich (1963) referred to a case of a colonic adenocarcinoma in man having a hypotetraploid mode as judged from measurements of the DNA values but showing only normal diploid cells in Pc after 28 days *in vitro* *Spriggs et al* (1962) found an altered chromosome picture in Ds from six cases of human carcinoma *in situ* of the cervix uteri However *Richart & Corfman* (1964) found a normal diploid stemline in five similar cases but only studied in Pcs These are two additional examples where stromal overgrowth could be the explanation for the discrepancy between D and Pc and actually Stich expressed this opinion in his case

There are also some related cases among animal tumours Thus *Hsu & Kellogg* (1960) briefly referred to a chromosomal study of a primary culture of Novikoff s hepatoma of the rat where normal diploid cells suddenly dominated the picture in Pc As stated this was certainly an example of stromal overgrowth *in vitro* Further Hsu quoted by *Basrur & Gilman* (1963) observed that normal rat cells show a selective advantage over rat tumour cells in culture The latter authors reported chromosomal studies of two strains derived from rhabdomyosarcomas of the rat In one cell strain a heteroploid stem line persisted all the time *in vitro* In the other strain there was a mixture of normal and heteroploid cells in the primary culture However about five months later the normal cells had outgrown the heteroploid stemline and this picture persisted during the reported observation time

However *Nichols* (1963) found no difference between D and Pc in the study of primary Rous sarcomas in the rat This is of great interest since his tumours were induced by the same virus strain as the present tumours and further the primary cultures were grown in the same medium Neither did *Hellstrom et al* (1962) find any differences between D and Pc in their investigations of primary and passed polyoma tumours in mice They used similar methods for cultivation and Pcs were performed after intervals varying between 24 hours and 4 days

Analyses similar to those performed in the present investigation will be necessary for interpreting the diverging results For the time being

it only possible to assume that different tumours in different species may differ as regard their capacity of outgrowth *in vitro*

SUMMARY

The present study a comparison between the results obtained in direct fixations from the tumour (D) and in fixations from primary cultures (Pc) was prompted by certain findings in a previous study of primary Rous sarcomas in mice In Pcs the original heteroploid stemline had often disappeared and been replaced by a normal diploid stemline Several of the primary sarcomas were studied during transplantation and in Pcs the same phenomenon a normalization appeared The present investigation showed that the discrepancy was due to stromal overgrowth *in vitro* This process usually became manifest after 20 hours cultivation and as a rule the picture remained unchanged for 10–14 days Subsequently tumour cells sometimes reappeared probably due to ceasing multiplication of stroma cells The stromal outgrowth *in vitro* was most apparent in primary sarcomas and during early passages In Pc from late passages *in vivo* the stromal overgrowth was sometimes reduced which might be due to a reduced stromal content and possibly to karyotype changes

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APPLICATION OF ENZYME HISTOCHEMICAL METHODS IN THE DIFFERENTIAL DIAGNOSIS OF MELANOMA AND LEIOMYOMA OF THE IRIS

By

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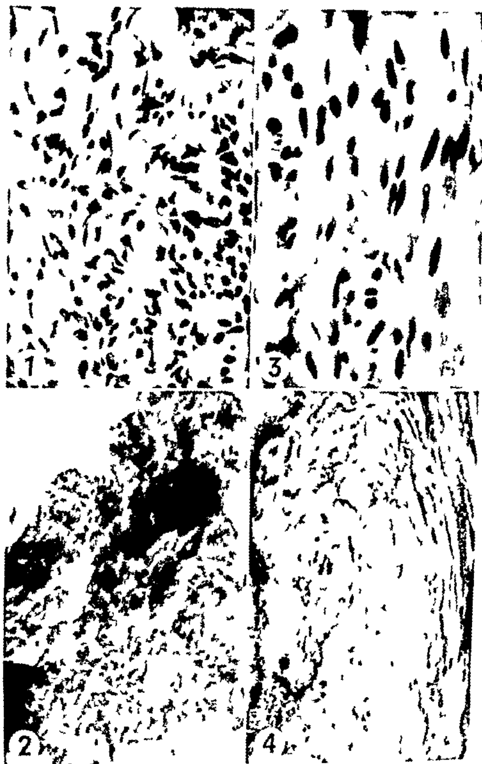
The difficulties in the histopathologic differential diagnosis between leiomyomas and malignant melanomas of the iris have been emphasized by *Rones & Zimmerman* (1958) and by *Hogan* (1964). There is no sharp clinical or pathologic distinction between these two groups in the majority of primary tumours of the iris. Usually gold impregnation, Mallory's phosphotungstic acid hematoxylin stain and the thionin inclusion stain have been recommended for the differential diagnosis between the neurogenic and myogenic tumors of the iris (*Boke* 1956). Unfortunately these stains do not always produce sufficient information for the differentiation between melanoma and leiomyoma.

In a previous paper by us (*Niemi & Tarkkanen* 1964a) it was shown that the sphincter and dilator muscles of the iris have histochemical characteristics of muscle tissue by showing activity of phosphorylase but contrary to the smooth muscle tissue elsewhere in the body they also contain intense acetylcholinesterase activity. Furthermore, two cases of leiomyoma of the iris (*Niemi & Tarkkanen* 1964b) showed strong acetylcholinesterase activity unlike leiomyomas of the skin (*Mustakallio et al.* 1963). Melanomas of the iris have not been studied histochemically.

It is the purpose of this paper to point out the value of some enzyme histochemical methods in the histopathologic evaluation of melanomas and leiomyomas of the iris.

MATERIAL AND METHODS

The material consisted of four cases of tumors of the iris removed by iridectomy. Immediately after the removal the specimens were embedded either in mouse kidney tissue or in gelatin and frozen on a tissue holder. Sections were cut at 20 to 40 µ in a cryostat, they were picked up on cover glass, let to dry for a couple of minutes and subsequently incubated for the demonstration of enzyme activity. Routine staining with hematoxylin-eosin, Masson trichrome and Mallory's phosphotungstic acid hematoxylin stains were performed on similar sections.


1
3
2
4

The methods for the demonstration of acetylcholinesterase (AChE) non specific holinesterase (ChE) monoamine oxidase (MAO) and phosphorylase activity were he same as employed in our previous study (Niemi & Tarkkanen 1964 a)

RESULTS

In two of the cases the tumor appeared both in hematoxylin eosin and Mallory s phosphotungstic acid hematoxylin stains as a leiomyoma (Fig 1 Cases 1 and 2 in Table 1) The two remaining tumors were very likely leiomyomas morphologically but phosphotungstic acid staining gave negative results (Fig 3)

With the aid of enzyme histochemical methods a good separation into two groups could be obtained in the entire series (Table 1) The first two leiomyomas showed high activity of specific cholinesterase (Fig 2) the activity of non specific cholinesterase being weak and localized to some coarse fibres in the tumor A weak monoamine oxidase activity could be demonstrated in the tumor cells and also the amylo phosphorylase activity was clearly demonstrable Since the pinkish colour of this reaction is difficult to demonstrate in black and white film no photomicrographs are available

TABLE 1

Degree of Histochemically Demonstrable Enzymatic Activity in a Series of Four Tumors of the Iris

	AChE	ChE	MAO	Phosphorylase
Case 1	+++	(+)	+	+
Case 2	++	(+)	+	+
Case 3	—	—	+	—
Case 4	—	—	±	—

— no activity
(+) uneven slight activity
From + to +++ slight to intense activity

The remaining two tumors belong histologically to the melanoma leiomyoma group (Fig 3) but could not be classified to either of these types definitely These tumors showed neither cholinesterase nor phos phorylase activity (Fig 4) A weak monoamine oxidase activity was present

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Figs 1 4

The Figures 1 and 2 ref r to C se 1 nd F u s 3 and 4 to Case 3 Table 1

Fig 1 Tumor of the iri a seen in hem t xylin eosin staining frozen sect on × 800

Fig 2 The tumor how n intense AChE ti ity wh ch appears to be located to the tumor cells 480

Fig 3 Tumor of the i omp s d with long lender nuclei and ill-def n d cytoplasm Hem t x l n e sin ozen section × 800

Fig 4 AChE activity n th tumo appears negative Compare the AChE a ti ity with the inten a t ty een in Fig 2 × 480

DISCUSSION

Of the four iris tumors presented two were classified as typical leiomyomas with routine histological stains and the two others were considered as being probably leiomyomas although no myofibrils were detectable

The muscular nature of the former tumors was revealed histochemically by the presence of phosphorylase activity In terms of strong acetylcholinesterase activity however the tumors differed from other leiomyomas of the body (*Mustakallio et al* 1963) but accepting their origin from the sphincter or the dilator muscle of the iris the findings are in agreement with the results of the normal iris muscles (*Niemi & Tarkkanen* 1964a) The histochemical observations demonstrated on the other hand that the two other tumors did not exhibit characteristic features of an iris leiomyoma and belong therefore to melanomas Recently a melanoma of the ciliary body managed through our procedure revealed similar results as these iris tumors

The tumors of smooth muscle origin in the iris may show on the one hand a muscle pattern and on the other hand a neuroepithelial pattern and are therefore different from leiomyomas in other areas of the body as also suggested by *Reese* (1963)

A very interesting tumor of the iris of uncertain histogenesis has been described by *Duke & Dunn* (1958) No myofibrils were identified although the smooth muscle origin of the tumor was considered

Rones & Zimmerman (1958) have emphasized that the tumors of the iris present a problem rarely encountered in those of the posterior uvea They found it difficult to make a differential diagnosis between melanomas and leiomyomas of the iris although theoretically these tumors belong to different groups They were able to divide their tumors of the iris into two groups The first one included those with cytology characteristic of the nevus melanoma and leiomyoma types and the second group those capable of metastasis

Apparently the application of enzyme histochemical methods may render more information of the nature of the tumors of the iris than what can be obtained by conventional staining methods On the basis of our results at the Helsinki University Eye Hospital all tumors of the iris are routinely being frozen fresh immediately after the removal and processed through the enzyme histochemical procedure in addition to conventional staining methods

SUMMARY

Four tumors of the iris removed by iridectomy and belonging to the melanoma leiomyoma group were frozen fresh immediately after the removal and stained by hematoxylin eosin Masson s trichrome and Mallory s phosphotungstic acid hematoxylin stains In addition acetyl cholinesterase (AChE) non specific cholinesterase (ChE) mono amine

oxidase (MAO) and phosphorylase activity of the tumors were demonstrated by enzyme histochemical methods Two of the tumors showed myoglial fibres an intense AChE activity weak ChE as well as MAO activity but a clearly demonstrable phosphorylase activity In the other two tumors no myoglial fibres were demonstrable There was no AChE or ChE nor phosphorylase activity but a weak MAO activity was present It is concluded that application of enzyme histochemical methods will render more information about the nature of the tumors of the iris which very often present diagnostic problems rarely encountered in the tumors of the posterior uvea

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THE MONONUCLEAR CELL INFILTRATE IN ALLERGIC CONTACT DERMATITIS

2. *Selective Accumulation of Cells from the Bone Marrow*

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The characteristics of the mononuclear cell infiltrate in allergic reactions of the delayed type have been studied in several previous investigations, most of which have employed transfusion of labelled cells between animals. In this way the immunological specificity of the infiltrating mononuclear cells has been studied with positive results (11, 16) contrasting with negative ones (2, 6, 8, 10, 20). The source of the infiltrating cells has also been investigated using transfusion of labelled cells. *Kosunen* (10, 11) found that cells from lymph nodes regional to the area of sensitization with tuberculin accumulated in delayed hypersensitivity reactions in a higher degree than cells from non-regional lymph nodes and spleen. Thymic cells were not found at all. However, the effects of the handling of the cells during preparation of the cell suspensions, the significance of the host defense mechanisms (17) and the influence on the distribution pattern of the cells within the recipient caused by the rapid addition of a substantial number of cells to the circulation must be regarded as unsettled.

Attention has therefore been focused on experiments where the cells have been labelled and traced within the same animal. From those experiments the following picture of the cellular infiltrate in the delayed reactions of tuberculin and/or contact type can be deduced. The cells belong mainly to the lymphoid series (12, 23). Most of them are haematogenous, have recently proliferated and show a large proportion of macrophages and large lymphocytes (2, 9). In contact allergic reactions, cells labelled on the 4th day after sensitization have an increased mitotic activity (13). The mitotic activity of the infiltrating lymphoid cells in general does not seem to be correlated to the different stages of the development of the reactions as judged from the data of *Kosunen et al.* (9) and *Groth* (7). Cells formed early in

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nique as for H3-TdR After the final injections these latter animals received intra peritoneall the same amount of H3-TdR (i e 20 µc) as the animals labelled locally in the bone marrow

In a preliminary experiment three animals labelled locally in the bone marrow were killed ½-1 hour after the injections for estimation of the efficiency of the bone marrow labelling and the degree of general labelling

Testing

The testing was performed on day 10 at 8 a m i e 20 ± 2½ hours after labelling (21) The tests were applied on the hind part of the right midflank where the fur had been removed the day before by an electric clipper and by an electric shaver at least half an hour before testing 20 µl of a 0 20 per cent alcoholic solution of DNCB was applied with a micropipette to an area of 2 cm (= 20 µg/cm a dose shown to be atoxic in several tests on different guinea pigs during the last years)

Biopsies Histologic and Autoradiographic Technique

48 hours after testing the material under study was collected Skin biopsies ere taken from the te t sites and from untreated control skin taken cranially or caudally from the test site in every second animal of the two labelling groups

The skin biopsies were fixed in carbonate buffered formalin dehydrated in alcohols cleared in xylene and embedded in paraffin The sections were cut with a microtome setting of 5µ Sections from both biopsies of each animal were placed on the same slides their positions on the slides being changed alternatively A skin section from nonlabelled (cold) animals was also mounted on each slide

For autoradiographs Kodak AR 10 tripping film was used After an exposure time of 4 weeks the slides were developed and stained with haemalum

The technical data given here are described in greater detail in a previous publication (13)

Evaluation of Autoradiograms

The criteria for the classification of cells as labelled were the same as those employed in a previous investigation in this series (13) i e lymphoid cells in corium with 3 grains or more were regarded as labelled Counting of 2 300 lymphoid cells in the corium from cold skin sections on 23 different slides from the experiment showed that 0 4 per cent of them had 3 grains or more

The skin sections were first screened for determination of the types of lymphoid cells showing labelling and their location in the skin (corium or epidermis) A systematic counting was then performed by a specially skilled technical assistant unaware of the experimental design From each of the two skin biopsies of every animal 1 000 lymphoid cells were counted in the upper parts of the corium i e as a rule not more than one and a half high power view field (1 000 ×) below the dermal epidermal junction These cells were counted from at least four sections from two different slides The labelled cells with different grain counts were separated from each other in the counting protocol

The lymphoid cells were identified as described by Groth (4) but macrophages (9) were also included in this group The intensity of the allergic reaction was measured by the lymphoid cell response (4)

RESULTS

The results from the systematic countings of the autoradiograms are presented in Table 1 and 2 In Table 1 the values of the control biopsies have been subtracted from the values of the test biopsies in order to compensate for possible minor differences in labelling efficiency between the animals Thus the difference between the values of test and control skin was used when comparing the animals The test biopsies show a significantly larger increase in the proportion of labelled lymphoid cells in the group labelled locally in the bone marrow

than in the group labelled intraperitoneally ($p<0.05$) The mean grain count values over the labelled lymphoid cells in test skin of the former group have increased whereas the corresponding values in the intraperitoneally labelled group have decreased (differences between the values significant $p<0.01$) In Table 2 the absolute uncorrected figures for the mean grain counts and the maximal individual grain counts are given The latter figures are very similar for both control and test skin from the intraperitoneally labelled animals and for control skin from the animals of the bone marrow labelled group Only the figures for the test skin in the bone marrow labelled group are higher However in 3 of the 8 animals the maximal individual grain count is below the highest of those in the intraperitoneally labelled group (= 12 grains) This is due to the fact that only 1 000 lymphoid cells were counted in the corium This number represents but a small fraction of the cellular infiltrate and the more heavily labelled cells present may not be encountered in this counting On screening of the autoradiograms however 12 grains was the highest number found in the intraperitoneally labelled group whereas cells with at least 30 grains were found in test skin sections from every bone marrow labelled animal The lymphoid cell responses as a rule are low in this experiment but show a comparable distribution in the two animal groups They show no correlations to the number of labelled cells as far as can be judged from this limited material

TABLE 1

Autoradiographical Values (Numbers of Labelled Cells and Mean Grain Counts) Have Been Calculated from Skin Sections of Two Groups of Guinea Pigs Sensitized to DNCB and Labelled with H3–TdR Locally in the Bone Marrow or Intraperitoneally

	Test skin values minus control skin values			
	Local bone marrow labelling		Intraperitoneal labelling	
	No of labelled cells	Mean grain counts	No of labelled cells	Mean grain counts
	13	1 8	7	—0 3
	6	0.2	15	0 8
	10	1 2	2	0 0
	16	[illegible]	7	—1 5
	10	—0 7	— 3	—0 1
	8	1 4	—1	—0 1
	6	2 2	— 4	—0.3
	5	3		
Mean values	9 3	1 3	3 3	—0 [illegible]
SE	1 0	0 4	2 6	0 3
Ranges T	13–34	3 [illegible]–6 [illegible]	9 36	3 5–4.3
C	5–18	3 3 4 8	9 [illegible]1	3 [illegible]–[illegible]2

The differences between the values of test skin and those of untreated control skin are given in the table 1 000 lymphoid cells have been counted in the corium [illegible] ch of the two skin types from every animal C = control skin T = test skin

nique as for H^3-TdR. After the final injections these latter animals received intraperitoneally the same amount of H^3-TdR (i.e. 20 µc) as the animals labelled locally in the bone marrow.

In a preliminary experiment three animals labelled locally in the bone marrow were killed ½-1 hour after the injections for estimation of the efficiency of the bone marrow labelling and the degree of general labelling.

Testing

The testing was performed on day 10 at 8 a.m., i.e. 20 ± 2½ hours after labelling (21). The tests were applied on the hind part of the right midflank, where the fur had been removed the day before by an electric clipper and by an electric shaver at least half an hour before testing. 20 µl of a 0.20 per cent alcoholic solution of DNCB was applied with a micropipette to an area of 2 cm² (= 20 µg/cm², a dose shown to be atoxic in several tests on different guinea pigs during the last years).

Biopsies, Histologic and Autoradiographic Technique

48 hours after testing, the material under study was collected. Skin biopsies were taken from the test sites and from untreated control skin taken cranially or caudally from the test site in every second animal of the two labelling groups.

The skin biopsies were fixed in carbonate buffered formalin, dehydrated in alcohols, cleared in xylene and embedded in paraffin. The sections were cut with a microtome setting of 5µ. Sections from both biopsies of each animal were placed on the same slides, their positions on the slides being changed alternatively. A skin section from nonlabelled ("cold") animals was also mounted on each slide.

For autoradiography Kodak AR 10 stripping film was used. After an exposure time of 4 weeks the slides were developed and stained with haemalum.

The technical data given here are described in greater detail in a previous publication (13).

Evaluation of Autoradiograms

The criteria for the classification of cells as labelled were the same as those employed in a previous investigation in this series (13), i.e. lymphoid cells in corium with 3 grains or more were regarded as labelled. Counting of 2 300 lymphoid cells in the corium from "cold" skin sections on 23 different slides from this experiment showed that 0.4 per cent of them had 3 grains or more.

The skin sections were first screened for determination of the types of lymphoid cells showing labelling and their location in the skin (corium or epidermis). A systematic counting was then performed by a specially skilled technical assistant unaware of the experimental design. From each of the two skin types of every animal 1 000 lymphoid cells were counted in the upper parts of the corium, i.e. as a rule not more than one and a half high power view field (1 000 ×) below the dermal-epidermal junction. These cells were counted from at least four sections from two different slides. The labelled cells with different grain counts were separated from each other in the counting protocol.

The lymphoid cells were identified as described by Froth (4) but macrophages (9) were also included in this group. The intensity of the allergic reaction was measured by the lymphoid cell response (4).

RESULTS

The results from the systematic countings of the autoradiograms are presented in Table 1 and 2. In Table 1 the values of the control biopsies have been subtracted from the values of the test biopsies in order to compensate for possible minor differences in labelling efficiency between the animals. Thus the difference between the values of test and control skin was used when comparing the animals. The test biopsies show a significantly larger increase in the proportion of labelled lymphoid cells in the group labelled locally in the bone marrow

general labelling is very slight Intraperitoneal injection of the same amount of marker is expected to give a more pronounced general labelling not favouring the bone marrow cells If the bone marrow cells contribute to the mononuclear infiltrate at the test site the test biopsies from the bone marrow labelled group should contain lymphoid cells with a maximal grain count exceeding that of the lymphoid cells in the intraperitoneally labelled group If there is not only a random contribution but also a selective accumulation of bone marrow cells in the cellular infiltrate of the test site the difference between the numbers of labelled lymphoid cells in the test skin and in the control skin should be larger in the group labelled locally in the bone marrow than in the intraperitoneally labelled group provided the percentage of labelled lymphoid cells is comparable in the two groups

Between ½–1 hour after local bone marrow labelling the cells in a control marrow in the same animal showed a grain count of 6 at the most The mean grain count in this population is obviously very low If cells from this population accumulate in the skin without mitoses after the labelling however part of them might be registered as labelled This would make the mean grain count over the labelled lymphoid cells low despite the presence of some heavily labelled cells If mitoses of these lymphoid cells not locally labelled occur between labelling and biopsy they will not be detectable as labelled Hence only cells coming from the locally labelled bone marrows will be registered as labelled *i e* the mean grain count would be high compared with this value in the intraperitoneally labelled group This argument would hold even if there was only a random accumulation of bone marrow cells However if this higher mean grain count occurs together with a percentage of labelled lymphoid cells at least as large in the group labelled locally in the bone marrow as in the intraperitoneally labelled group it favours a selective accumulation of the bone marrow cells

The results show that the differences between the numbers of labelled cells in test skin and in control skin are larger in the group labelled locally in the bone marrow than in the intraperitoneally labelled group The differences between the mean grain count values are also more pronounced in the former group than in the latter at the same time as the proportion of labelled cells is similar in the two groups According to the reasons given above both these results favour a selective accumulation of bone marrow cells to the test site

The increase in mean grain count values in the test biopsies from the bone marrow labelled group contrasts with the slight decrease of these values in the intraperitoneally labelled group This latter decrease is in accordance with the results obtained in an earlier work (13) where it was made probable that the mitotic activity in some of the cells infiltrating the allergic reaction was elevated This would give a dilution of the label It is possible that this increased mitotic activity

is present also in the locally labelled bone marrow cells If so the initial grain counts are probably so high that in this group of animals the diluting effect of the mitoses is less pronounced than in the intra peritoneally labelled group

The absence of labelled small lymphoid cells in this investigation is noteworthy *Kosunen et al* (9) found an increasing proportion of labelled macrophages in the test lesions up to 48 hours the latest time chosen for biopsies in their experiment These results were interpreted as indicating a transition of lymphocytes to macrophages It is possible that such transitions (for ref see 19) explain the absence of labelled small lymphocytes observed in the present experiment and the sparse ness of such cells in the experiment of *McCluskey et al* (2) in which labelling was performed during the 36 hours preceding testing In any case the available data do not permit any conclusions with regard to the question of which cell type is labelled initially and later accu mulates in the test reactions

The lack of heavily labelled lymphoid cells in the corium of control skin indicates that the lymphoid cells labelled in this experiment are not a normal constituent of the skin They seem to accumulate only at the sites of inflammatory reactions If this accumulation is an im munologically specific answer or not is open to question The results of *Blazkovec Sorkin & Turk* (1) may favour a special significance of the macrophages These authors found that local passive transfer of cells from peritoneal exudate (which contained 25 per cent macro phages) resulted in delayed hypersensitivity to tuberculin as com pared with transfusion of lymph node cells when the transfusion attempts failed Cells of the macrophage type have been found to take up antigen in delayed reactions both in skin (3 18) and in the regional lymph node (15) However the localization of the antigen was not influenced by sensitization to that antigen (3 18) In both specific and non specific lesions cells labelled shortly before testing accumulated in the same degree (9) These latter findings indicate that at least the majority of these cells respond to a non immunological stimulus

From the present experiment it is clear that cells from the bone marrow compose part of the mononuclear cell infiltrate in allergic contact dermatitis About 3 days after labelling and 48 hours after application of the test they appear mostly as macrophages or large lymphocytes Most of the labelled cells in the corresponding group injected intraperitoneally belong also to the same group of cells This is true also for the cells labelled in the previously cited work of *McClus key et al* (2) in both allergic contact dermatitis and delayed reactions of tuberculin type These similarities justify the tentative conclusion that the large lymphoid cells in delayed hypersensitivity reactions *in general* are derived partly from the bone marrow

SUMMARY

Two groups of guinea pigs sensitized to DNCB were labelled with tritiated thymidine either intraperitoneally or locally in the bone marrow. Autoradiographic analysis of sections from contact allergic reactions and from untreated control skin from each animal in the two groups was performed. Bone marrow cells labelled about 20 hours before epicutaneous testing were found to accumulate selectively in the 48 hour test reactions whereas no certain bone marrow migrants were found in control skin. Most of the labelled cells had the morphology of macrophages and large lymphocytes in both groups; thus no labelled small lymphocytes were found.

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CHOLERA FILTRATES AND AMMONIUM ION

Inhibitory Effect on Short Circuit Current of the Isolated Frog Skin

By

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Received 31 viii 66

In 1960 it was shown by *Fuhrman* (4) that an inhibitor of the active sodium transport on the isolated amphibian skin was present in batches of a commercial preparation of cholera filtrate The inhibitor was however not found in all batches of this filtrate (produced by Philips Duphar Holland) These observations have been confirmed (10)

Many attempts have been made to produce filtrates of *in vitro* cholera cultures with inhibitory effect primarily without success (5) but recently the production of active preparations have been reported (1) The present paper reports on the apparently successful production of filtrates capable of inhibiting the sodium pump

During an analysis of the occurrence of the inhibitory factor in our filtrates there seemed to be a correlation between the pH of the filtrates and their inhibitory activity The experiences reported by *Neptune & Mitchell* (11) that ammonia might have an inhibitory effect on the active sodium transport through the isolated frog skin therefore suggested a determination of the ammonia contents of the filtrates used for the frog skin experiments as well as experiments to determine the effect of ammonia on our frog skins

The effect of vasopressin on the active sodium transport in the frog skin inhibited by cholera filtrates as described earlier (10) has been used throughout the present studies

MATERIAL AND METHODS

Active filtrates ha e be n produced mainl w h the strain of V *cholerae* designated 4Z which has been us t for the producti n f the commercial f ltrate Other stra ns ha e also been used a for example the f e hl solated strain of V *cholerae* No 51631 This strain an l s ral other we e obt ned from the Pakistan SEATO

The study upon wh ch this pap based r ceived financial support f om the W rld Health Organi at

We would like to thank Mr E L lb lle fo his skilful techn cal ssistan e

Cholera Research Laboratory[1] Dacca East Pakistan Strain 51681 was isolated from a cholera patient on November 14th 1964 A strain of *E coli* designated U4 with the antigen formula O4 K3 H5 has also been used for some experiments

The media used were peptone solutions in concentrations of 5 or 10 per cent Difco Bacto Peptone of several production numbers as well as Brunnengräber (Lubeck) peptone have been used In some cases 0 5 per cent sodium chloride was added to the 5 per cent peptone but no effect of this addition was observed The incubation time varied from 20 to 350 hours but was generally between 100 and 200 hours The incubation took place in an air incubator at 37 C

The majority of the filtrates were made from cultures grown in 1 litre Erlenmeyer flasks containing 50 ml medium to give a high ratio of surface to volume (3) The media were inoculated with a loopful of bacterial culture from a solid medium The cultures were centrifuged at 29 000×g for 20 minutes after completion of the incubation and finally filtered by suction through membrane filter (*Schleicher & Schull Nr 1121) Having passed sterility control the filtrates were distributed in* small rubber stoppered vials and kept at —20 C Several recent batches of Cholera Filtrate Philips Duphar have been examined[2] The contents of the ampoules were dissolved in 10 ml distilled water

When ammonia was used a 1 14 M NH_3 solution was adjusted with concentrated hydrochloric acid to pH 8 5 This solution was added to cholera filtrates peptone solutions or directly to the solution bathing the outside of the skin in experiments made to investigate the effect of the ammonium ion on the sodium pump

Filtrates of stools from cholera patients were obtained from the Pakistan SEATO Cholera Research Laboratory Dacca East Pakistan The stools had been sterilized by membrane filtration shortly after collection and were kept at +4 C Since arrival at this laboratory they have been kept at – 20 C Details and bacteriological findings are given in Table 3

The vasopressin used was Insipidin from A/S Alfred Benzon Copenhagen or *Vasopressin synth Sandoz* The dose was *0 2 or 1 I U added to the solution bathing* the inside of the skin

The experiments were performed with the appartus described by *Ussing* (13) The short circuit current was followed by frequent adjustment to zero of the potential over the skin

The frogs used were mainly of the species *Rana temporaria* but also other non specified species as obtained in the sendings of frogs from southern Germany were used In a few instances the abdominal skin from one frog was divided in halves and used in two parallel experiments The experience was that this gave a very good control as the halves gave very similar results

The cholera filtrates were added to the anatomical outside of the frog skin in amounts varying from about 0 5 ml to 10 ml The same amounts of frog Ringer were added to the inside of the skin to make up for differences in hydrostatic pressure in the two compartments of the frog skin apparatus

Measurements of the inhibition of the short circuit current of the isolated frog skin is not a method well suited for quantitative estimations The variability is great and the measurements are slow particularly because considerable time is required before the short circuit current stabilizes In the present experiments it has generally been necessary to secure that the short-circuit current has been stable over a period of at least 20–30 minutes before the skin was found suitable for experimentation This limited usually for the present purposes the working capacity of a frog *skin apparatus to one or at the most two skins in one working day*

Estimation of pH was made electrometrically Ammonium content was estimated by the microdiffusion method of Conway using the boric acid HCl modification (?) This method will probably also measure volatile amines which might be present but the results are expressed as NH_3

An attempt has been made to obtain a rough quantitative measure of the potencies of different cholera filtrates by using a scoring system as follows

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1 We are grateful to Dr *Benenson* and Dr *Craig* for their kind assistance in providing a number of freshly isolated cholera strains and filtrates of stool from cholera patients

2 We are grateful to Dr *Hertberger* Philips Duphar for supplying the batches of Cholera Filtrate

Reaction	Des ription of variation in short-circuit current follow ing addition of cholera filtrates	Figure
Total	Initially there might be a quick decrease immediately fol lowed by an increase The rise in current might continue over the initial level but is quickly followed by a steep decrease within 20 minutes after the application of the sample No reaction to vasopressin	1
Average	An initial current decrease immediately followed by an increase is sometimes observed The increase may con tinue above the initial current level but is followed b a decrease of moderate steepness Moderate positive reac tion to vasopressin	2
Doubtful	The initial current dive might be b erved always fol lowed b an increase in the current ascending consider ably above the initial level A subsequent slow decrease does not al ays reach the level of the current observed before the additi n of filtrate Positive reaction to vaso pressin	3
None	The initial dive in current might be observed followed either b no changes in current or a considerable increase The current remains at the level achieved and reacts strongly to vasopressin	4

We have not used the percentage decrease in current after application of the material to be tested because the reaction seems to be composed of two competitive reactions one causing an increase the other causing a decrease in the short-circuit current

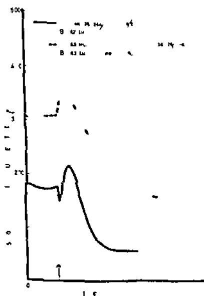


Fig 1
"Total rea t n

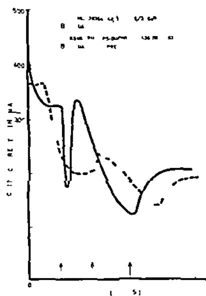


Fig 2
"Averag reaction

TABLE 2

Cholera Filtrates Produced with 10 per cent Bacto Peptone

Filtrate No	Incubation (hours)	Dose (ml)	Reaction	Organism Spec	Organism Strain	pH	NH3 mM/l outside sol	Date of exp
110464/90	90	10	Total	V chol	47		31	21/4/64
–	–	5	Average	–	–		15	–
–	–	2 5	Average	–	–		8 5	–
–		1 25	Average	–	–		4 5	22/4/64
	–	0 625	None	–	–		2 3	–
010664/120	120	5	Average	*E coli*	U4	8 6	20	2 3 65
–	–	2	Average	–	–	–	9 5	2 8 64
–	–	2	Average	–	–	–	–	11/8 64
–	–	2	*Doubtful*	–	–	–	–	1/3/65
–	–	1	Average	–	–		4 9	3 6 64
–	–	1	Average		–	–	–	4 6
–	–	1	Average	–		–		–
–	–	0 5	None	–		–	2 5	2 6/64
080664/120-U4	120	10	Total	–	–	8 61	40	9/6 64
–	–	5	Total	–	–	–	20	2 /11/64
–	–	*2*	Average		–	–	8	10/6 64
–		2	Average	–	–	–	8	–
–	–	2	None	–	–		8	26/11/64
190365/168	168	5	Total	V *chol*	*47*	8 42	18 2	4/4 65
–	–	5	Average	–	–	–	–	8/4/65
–	–	5	Average	–	–	–	–	12/4 65
	–	5	Total	–	–	–	–	13/4 65
–	–	5	Total	–	–		–	14/4 65
–	–	5	Total	–	–		–	20/4 65
140465/166	166	5	Total	–	–	8 38	15 5	23/4 65
–	–	5	Total	–				26/4/65
–	–	5	Doubtful		–	–	6 2	27/4/65
–	–	5	Doubtful	–	–	–	–	3 6/65
080465/314	314	5	Total	–	–	8 06	15	28/4 65
	–	5	*Average*	–	–		–	9/4 65
010565 427075	168	5	Total	V *chol*	51681	8 63	20 1	11/5 65
–	–	2	Total	–	–	–	9 0	13 5 65
–		1	Doubtful	–	–	–	4 7	1 /5 65
010565 471192	168	5	Total		–	8 41	21 3	17 5 65
–	–	2	*Doubtful*		–	–	9 5	18 5 65
110565-427075 Rug	168	5	*Average*		–	8 71	20	5 5 65
–	–	2	Total	–			9 2	26 5 65
–	–	2	*Average*	–				29 6 65
–	–	2	Total			–		30 6 65
–	–	2	Total	–		–	–	1/1 65
–	–	1	*Doubtful*	–	–	–	4 8	28 5 65
		1	None	–		–		29 5 65
–	–	1	*Doubtful*		–			31/7 65

TABLE 2 (cont.)

Filtrate No	Incubation (hours)	Dose (ml)	Reaction	Organism Spec	Organism Strain	pH	NH_3 mM/l outside sol	Date of exp
260565-472005	168	5	Total	–	–	8.68	18.3	3 6 65
–	–	2	Average	–	–	–	8.1	4 6 65
–	–	1	Average	–	–	–	4.2	8 6 65
–	–	0.5	Doubtful	–	–	–	2.2	9 6 65
–	–	0.5	Doubtful	–	–	–	2.2	10 6 65
260565 904659	168	1	Total	–	–	8.59	4.3	11 6/65
–	–	0.5	None	–	–	–	2.2	14/6 65
–	–	0.5	Doubtful	–	–	–	–	15 6 65
–	–	0.5	Doubtful	–	–	–	–	17/6 65
150665-472005	168	1	Average	–	–	8.57	4.2	24 6/65
–	–	1	Average	–	–	–	–	25 6 65
–	–	1	Average	–	–	–	–	26 6 65
190665-472005	168	1	Average	–	–	8.59	4.8	2/7/65
–	–	1	Average	–	–	–	–	2 7/65
–	–	1	Average	–	–	–	–	19 7/65
–	–	1	None	–	–	–	–	22 7/65
–	–	0.5	None	–	–	–	2.4	21/7/65
020765-904659	168	2	Average	–	–	8.68	8.7	12/7 65

TABLE 3

Stool Filtrates from Cholera Patients

Stool No	Dose ml	Reaction	pH	NH_3 mM/l outside solution	Pathogen isolated
812	2	None	8.8	0.8	Inaba
1213	2	None	7.4	0.8	None
1214	2	None	8.2	2.1	None
1215	2	None	8.5	0.7	Inaba
890	2	None		0.4	Ogawa
917	2	None		0.8	Inaba
926	2	None		0.8	None
1016	2	Average		8.2	None

The first sending containing four stools (890, 917, 926 and 1016) was lost during transport for four months. The conditions under which these filtrates have been kept are not known.

seven with ammonia contents from 6 to 29 mM/l filtrate proved inactive when tested in 2 ml doses (0.5–2.1 mM/l outside solution). One filtrate contained 111 mM/l NH_3 and showed an Average reaction when tested in 2 ml doses (Table 3).

The addition of NH_3 was accomplished by using a NH_4Cl solution

containing 1.14 M/l NH_3 and adjusted to pH 8.5. The effects of different doses of this solution are listed in Table 5 and an example is shown in Figure 5. The correlation between the short circuit reaction and NH_3 content is in reasonably good agreement with the corresponding figures found in tests with the cholera filtrates.

TABLE 4
Philips Duphar Cholera Filtrate Batch P029

Date of experiment	Dose (ml)	NH_3 mM/l outside sol.	Reaction
9/3/1964	10	4	Total
12/2/1965	5	2	Total
10/2/1965	4	1.5	Total
31/1/1964	2	0.8	None
31/1/1964	1	0.4	Doubtful

TABLE 5
Ammonium Chloride Solution 1.14 M/l pH = 8.5

Experiment No.	Dose µl	Reaction	NH_3 mM/l outside solution
1	100	Doubtful	4.6
2	125	Average	5.7
3	150	Average	6.8
4	150	Average	6.8
5	300	Average	13.7
6	400	Doubtful	18.2
7	400	Total	18.2
8	400	Total	18.2
9	400	Total	18.2
10	400	Total	18.2
11	400	Total	18.2

A few experiments have been performed with cholera filtrates where NH_3 was removed by the addition of sodium hydroxide. After storage for 24 hours at reduced pressure over diluted sulphuric acid (10 per cent) whereby the NH_3 was removed by evaporation, the pH was returned to 8.2 by means of concentrated hydrochloric acid. By diluting with sodium chloride free frog Ringer solution the isotonicity was restored and the final volume was adjusted to twice the original.

By this procedure the NH_3 content was reduced from about 120 mM/l filtrate to about 18 mM/l original filtrate. At the same time the inhibitory effect disappeared.

Addition of NH_3 solution to obtain the original concentration of NH_3 restored the inhibitory effect.

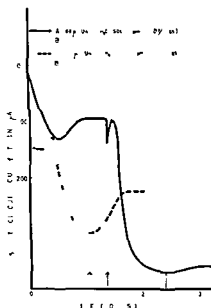


Fig 5
The effect of NH_3 on the isolated frog skin

DISCUSSION

The experimental demonstration of sodium pump inhibitors in Cholera Filtrate Philips Duphar (4) and in stools from cholera patients (6) supported the theory that the diarrhoea in cholera was caused by an inhibition of active absorption of sodium from intestinal lumen to blood

The inhibitory activity was however not always found in these preparations and many attempts to produce the inhibitor by growing V *cholerae* in different media were unsuccessful (5)

We have prepared active filtrates with 5 and 10 per cent peptone solutions using prolonged incubation With such preparations typical responses were regularly obtained using doses between 1 and 10 ml

A series of control experiments with uninoculated 5 per cent and 10 per cent peptone solutions have consistently shown only a rise in the short circuit current and no subsequent decrease

Attempts to develop a quantitative method for estimating contents of inhibitor were unsuccessful due to the variability of the test system Therefore the scoring system described was adopted The isolation or concentration of the inhibitor failed Even the very gentle gel filtration method was not successful The inhibitory effect disappeared after passage through a column of Sephadex G 25 and it was not possible to elute any inhibitory principle from the columns by various eluents

Considerable difficulty was encountered due to changing sensitivity of the frog skins from winter to summer. It seems to be that the sodium pump is almost unsusceptible to certain agents during winter but that during spring the sensitivity is rising simultaneously with the start of the mating activity.

When tested in the spring and summer seasons filtrates produced on 5 or 10 per cent peptone media proved active in inhibiting the short circuit current. Roughly the activity was doubled by increasing the peptone concentration from 5 to 10 per cent. The doses necessary to cause Average reaction were 3.5 and 2.0 ml respectively. For comparison the dose employed of the originally used commercial filtrates (10) was only 0.5 ml.

During the Cholera Symposium held in Honolulu in January 1965 it was described (11) that ammonium ions in a concentration of about 4 mM/l in the solution bathing the outside of the frog skin had an inhibitory effect on sodium transport as measured by the short circuit current.

A similar effect has been reported earlier by *Huf* (7, 8) who presented data showing the inhibitory effect of NH_4 ions at pH = 7.5 when substituting K ions in the solution bathing the outside of the skin.

It appears that the influence of ammonia on the active sodium transport when applied in ordinary frog Ringer solution depends on the pH of the solution so that inhibition is observed regularly only at pH higher than 8 (9).

We examined the NH_3 content of many of our cholera filtrates using the method of Conway. As already described and as may be seen from the Tables 1 and 2 a good correlation was found between the contents of ammonia or ammonia like substances in cholera filtrates and their inhibitory effect on the active sodium transport.

It was also shown that NH_4 ions (or NH_3 molecules) in the form of a solution of ammonia in hydrochloric acid with a pH of 8.5 would cause an inhibition of the short circuit current very similar to the effect of cholera filtrates.

Human cholera stools with low NH_3 content did not inhibit the short circuit current whereas such inhibition was observed with a single stool filtrate with higher NH_3 content.

With a recent particularly active Philips Duphar cholera filtrate inhibition was observed after applying 4 ml filtrate which is equal to 1.5 mM NH_3 per litre outside solution. The ammonia content is considerably lower than that found in filtrates of our own preparation. However the amounts of filtrate necessary to yield a total response were higher than the dose used with the filtrates tested earlier (10).

The question about the identity of the inhibitor found in our filtrates (which we believe is ammonia) and the inhibitor found in the Philips Duphar filtrates previously tested cannot be finally settled as nothing is left of these old filtrates.

Certain arguments may be formulated against the identity For ex ample the incubation time used for the production of the commercial filtrate is only 16 hours on semi solid media which in our experience is too short a time for the accumulation of sufficient amounts of am monia in liquid media Our observation with a recent Philips Duphar filtrate mentioned above where inhibition of the short circuit current was obtained with amounts of filtrate yielding as little as 1 5 mM ammonia per litre outside solution seems also to indicate that the inhibitor in this filtrate is of another kind than that found in ours Total inhibiting doses of our filtrates generally created an ammonia concentration from 10 to 20 mM NH_3 per litre outside solution in agreement with the concentration needed of a pure ammonium chloride to effect similar reactions on the frog skin The observation by *Fuhrman* (5) that the inhibitory activity resisted heating with alkali seems to exclude that the inhibitor in the Philips Duphar filtrates could be ammonia

On the other hand the reactions observed on the short circuited frog skin are very similar in all details The inhibitor is in both preparations able to pass a dialysis bag and it has in our hands been impossible to purify the inhibitor from either source by means of gel filtration

This in connection with the low specificity of the test system sug gests that the inhibition found in our cholera filtrates and perhaps also in stools from human cholera patients at least partly is caused by contents of ammonia or ammonia like substances

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PRECIPITINOGENS IN BETA HEMOLYTIC STREPTOCOCCI AND SOME RELATED HUMAN KIDNEY ANTIGENS

By

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Received 3 x 66

The recognition of acute glomerulonephritis as a sequela to a recent infection with nephritogenic streptococci has been well established by means of epidemiological studies (*Rammelkamp et al* 1952 *Rammelkamp* 1953 and *Wertheim et al* 1953) The nephro pathogenetic role of the streptococcus is however still a matter of controversy Besides a direct toxic or enzymatic action of streptococcal products such as streptolysin S (*Tan & Kaplan* 1962) or DPNase (*Bernheimer et al* 1957) various immunological explanations have been discussed (reviewed by *Cruickshank* 1963 *Dixon* and *Feldman* 1964 and *Cluff & Johnsson* 1965) Gamma globulin and complement (*Mellors & Ortega* 1956 *Freedman et al* 1960 *Freedman & Markowitz* 1962 and *Lachmann et al* 1962) as well as streptococcal antigens (*Seegal et al* 1959) and antigen antibody complexes (*Andres et al* 1966) have been demonstrated in glomeruli from patients with acute glomerulonephritis These findings may partially elucidate the immunopathogenetic mechanism of streptococcal induced glomerulonephritis

The antigenic composition of cell walls from beta hemolytic streptococci belonging to Lancefield s group A have been extensively examined (e g *Lancefield* 1954 *Krause* 1958 *Krause* and *McCarty* 1961 *McCarty* 1964) and the biological significance of these antigens has also been investigated (*Lancefield* 1954 *McCarty* 1964 *Holton & Schwab* 1966 *Abdullah & Schwab* 1966) Although the streptococcal antigens in culture filtrates (reviewed by *Halbert* 1964) have been intensively studied few reports are available concerning the intracellular streptococcal antigens (*Wilson & Wiley* 1963 *Wahl et al* 1965) That such studies might be of special importance in relation to the immunopathogenicity of streptococci in acute nephritis was illustrated by *Markowitz & Lange* (1964) and *Kaplan* (1964 1965) who could show antigenic relationships between human tissues and streptococcal cells

The aim of the present investigation was to investigate the intra

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cellular antigenic composition of streptococcal cells belonging to Lancefield s groups A C and G and to compare these antigens with those occurring in ordinary Lancefield extracts of cell walls as well as to antigenic factors derived from normal human kidney

MATERIAL AND METHODS

Bacterial strains Fourteen strains of beta hemolytic streptococci belonging to Lancefield s groups A C and G were investigated These strains are listed in Table 2 Primary human pathogenic strains are designated with type numbers The reference strain P 13846 (type 12 M+) was isolated from a patient with an acute glomerulonephritis in Bedale England 1963 This strain and the non nephritogenic type 12 streptococcus (strain 8300) were obtained from the Central Public Health Laboratory Colindale London The other streptococcal strains were kindly supplied by Dr Rebecca Lancefield The Rockefeller Institute New York In addition two pneumococcal strains (types 27 and 2 R) obtained from Statens Seruminstitut" Copenhagen were employed

Antigen Preparations

Streptococcal Intracellular material (IC) Transfers from 16 hours glycerine broth stock cultures of the streptococcal strains were grown in an antigen free medium under stabilized pH conditions at pH 6 8 as described by *Holm & Falsen* (1966) After 16 hours cultivation the cultures were centrifuged at 1500 ×g for 30 min at 4 C The bacterial cells were suspended in 10 volumes of buffered saline (M/15 phosphate buffer pH 7 0) centrifuged and washed three times in the same way The final bacterial sediment was suspended in 3 volumes of buffered saline sonicated in a Raytheon 200 watt 10 kc oscillator for one hour under chilling with cold tap water (<10 C) and subsequently freeze pressed five times in the x press described by *Edebo* (1960) After thawing the material was mixed with glass beads (7 mm) and shaken at 4 C for three hours decanted and centrifuged at 12 000 ×g for 30 min The supernates were designated IC (*e g* IC A12 (P 13846) IC A3 (S 84) etc) The protein content of the IC preparations varied between 4 1 and 6 0 mg/ml as measured by the method described by *Lowry et al* (1951) using lysozyme as a reference substance Three to five IC preparations from each strain were analyzed and the one with the most complete precipitinogenic composition was employed in the investigation

Lancefield extracts (CM) The bacterial sediments obtained after the preparation of the IC material were washed three times with distilled water and centrifuged at 12 000 ×g for 20 min These sediments were used in the preparation of Lancefield extracts (boiling at pH 2 0 for 10 min., followed by neutralization and centrifugation) The e preparations were designated CM (*e g* CM S 84 CM P 13846)

Streptococcal polyglycerophosphate antigen This preparation was obtained as a gift from Dr *Maclyn McCarty* for preparative procedure see his report (1959)

Human kidney antigen (K sup K sed) The source of these materials was the kidneys of a six week old boy who died of heart failure They were removed immediately after death washed with saline chilled in an ice bath and homogenized with 3 volumes of buffered saline in an Ultra Turrax at 24 000 rpm for three minutes under chilling After centrifugation at 3 000 ×g for 30 min a kidney tissue supernate (K sup) containing 3 mg protein/ml and a kidney tissue sediment were obtained This sediment was suspended in an equal volume of buffered saline (pH 7 4) and was designated K sed Both preparations were bacteriologically sterile

Human lung antigen was prepared from lung tissue of the same origin as the aforementioned kidneys using the procedure just described No streptococcal contamination was noted in this preparation

Pneumococcal intracellular antigen was prepared in the manner earlier described for streptococcal intracellular material

Antisera

Antistreptococcal serum (anti IC+EC) was produced in a sheep which had been immunized by repeated subcutaneous injections of IC material and culture filtrates

(EC) from the eight group A streptococcal strains using an adjuvant technique Each injection consisted of 0 5 ml pooled IC + 10 ml pooled EC the mixture was emulsified with an equal volume of paraffin oil Arlacel® (9 vol 1 vol) The immunization schedule implied weekly injections for 8 weeks followed by monthly injections for one year The antiserum bleeding employed in the present investigation was taken one week after the twelfth injection and was designated Anti IC+EC No antibodies could be detected in anti IC + EC against the medium used for cultivation of the streptococci Portions of this antiserum were absorbed with heated or unheated IC materials from different streptococcal strains The absorptions were carried out by adding an aliquot of the absorbing antigen to a portion of the antiserum incubating at 37 C for 1 hour and storing at 4 C overnight The mixture was centrifuged at 10 000 $\times g$ at 4 C for 30 min, and the supernate decanted If precipitins to the absorbing antigen were still detectable the procedure was repeated

Anti human kidney serum (anti K | P) Four rabbits were injected subcutaneously once a week for 8 weeks with an emulsion prepared by mixing 0 5 ml K sed with 0 5 ml paraffin oil – Arlacel A® (9 vol/1 vol) A booster dose of 0 5 ml K sed in 0 5 ml saline was given intravenously three weeks after the eighth injection and bleedings were performed one week later The sera after separation from the blood cells were repeatedly absorbed as described before with freeze dried pooled human plasma lacking demonstrable streptococcal precipitins The absorbed antisera were tested by the double diffusion in gel method and found free from antibodies against serum proteins in addition no antibodies against constituents of the streptococcal medium could be detected The serum which contained the largest number of precipitins when tested with K sup was chosen and was designated anti K | P

In some experiments the anti K | P absorbed with human AB(+) erythrocytes was used This absorption was performed by suspending a sediment of erythrocytes (1 volume) in anti K | P (2 volumes) This mixture was incubated for 1 hour at 37 C followed by centrifugation at 1 000 $\times g$ for 10 min

DPNase inhibition test Analyses for the detection of DPNase inhibiting capacity of IC materials from different streptococcal strains were made according to a technique recently described by *Holm & Kaijser* (1966)

Diffusion in gel analyses The double diffusion in gel method of *Ouchterlony* (1962) was employed in the gel chamber modifications described by *Wadsworth* (1962) and *Holm* (1965) The interpretation of the comparative precipitation patterns was made according to the principles described by *Ouchterlony* (1962)

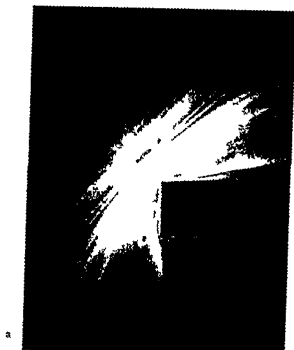
Immunoelectrophoresis Immunoelectrophoresis and comparative immunoelectrophoresis were performed according to the techniques described by *Wadsworth & Hanson* (1960) To obtain optimal separation of the streptococcal antigens the antigen reservoir had to be placed 4 cm from the cathode and the separation was made on 9 × 12 glass plates applying a field strength of 7 V per cm for 80 min

Registration of precipitates Photographic records of the precipitation patterns were made by means of Polaroid panchromatic film reproductions according to *Wadsworth* (1963)

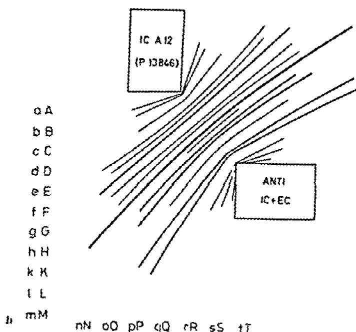
EXPERIMENTS AND RESULTS

Reference Streptococcal Precipitation Spectrum

To facilitate comparison of the various antigenic materials a reference streptococcal immune system was established The reference antigen consisted of the IC A12 (P 13846) preparation and the reference antiserum was the polyvalent sheep anti IC + EC Fig 1 a is a photograph of the spectrum developed by the undiluted reference antigen and the reference serum Additional precipitation lines were observed when one of the reactants was diluted The reference precipitation spectrum formed by these reactants consisted of eighteen precipitation bands s Fig 1 b By dilution of the reference antigen or the reference serum it could be verified that these bands represented separate immunological systems The lines were designated *aA – tT*



a



b

Fig 1

The reference streptococcal immune spectrum

a) Photograph of the precipitation pattern obtained with undiluted reference reactants. Upper basin: reference antigen IC A 12 (P 13846); lower basin: reference immune serum anti IC+EC.

b) Schematic drawing of the separate immune systems comprising the total reference spectrum

labelling from the antigen basin The antigenic factors are represented by small letters and the corresponding antibodies by capitals The most dense and rapidly appearing line in the precipitation pattern was the *kK* line while the *a* 1 – *eE* lines were fainter less distinct and appeared late in the diffusion analyses

The influence of heat on the precipitinogens in the reference antigen was examined by testing samples after heating them at 60 80 100 or 120 C for 30 min The heated preparations were tested with the comparative double diffusion in gel technique employing the reference streptococcal immune system The results of these analyses are shown in Table 1 As may be seen the *c g* and *k* factors could not be detected after heating the reference antigen to 60 C for 30 min while the factors *n p* and *q* were still demonstrable after heating to 120 C for the same period of time

The reference antigen was also treated with trypsin (1 mg ml Difco 1 200) for 60 min at 37 C pH 8 0 the enzyme action was blocked by addition of trypsin soy bean inhibitor (NBC 1 mg ml) It may be seen in Table 1 that the factors *c–f h l n o q* and *r* were not destroyed by the trypsin treatment and *n* and *q* resisted heating at 120 C for 30 min followed by exposure to trypsin

Immunological Comparison of the Different IC Preparations with the Streptococcal Reference Antigen

The streptococcal IC preparations were analyzed with the established reference immune system by means of the comparative diffusion in gel plate technique The results of these analyses are given in Table 2 The relative concentration of the identified factors is not indicated in the table As some of the lines in the reference spectrum were faint and did not extend far into the area of interference the coalescence of these lines with those formed by the reference immune serum and the IC preparation was sometimes uncertain Such reactions are indicated by question marks It may be seen in the table that streptococcal strains which are considered as primary human pathogenic *i e* the group A strains types 7 and 20 in group C and the T 16 strain of group G had the *a b g* and *k* factors These factors could not be detected in the other three strains tested Factor *f* was only found in group A streptococci Factors *n* and *q* were present in all the *A C* and *G* streptococcal strains and also in the two pneumococcal strains tested (pneumococcus types 27 and 2 R) Factors *c* and *l* were detected with certainty only in the antigen preparation from the reference strain and factor *p* only in the type 3 and the type 16 strain in addition to the reference strain The factors *e* and *r* were found in all the group A C and G streptococcal strains tested Similar results were obtained concerning the *s* and *t* factors although some uncertainty existed with regard to strains T 20 and K 39 Factor *d* was found in all group A and G strains but only with certainty

TABLE 1

Resistance of Precipitinogens in the Reference Antigen to Heat and Trypsin

Reference antigen	Precipitinogens demonstrated																	
	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	r	s	t
Untreated control																		
60 C 30 min	+	+	—	+	+	+	—	+	—	+	+	+	+	+	+	+	+	+
80 C, 30 min	—	—	—	—	—	+	—	+	—	—	(+)	+	—	+	+	+	+	+
100 C, 30 min	—	—	—	—	—	—	—	+	—	—	—	+	—	+	+	+	—	—
120 C 30 min	—	—	—	—	—	—	—	—	—	—	—	+	—	+	+	—	—	—
Trypsin 60 min	—	—	+	+	+	+	—	+	—	+	—	+	+	—	+	+	—	—
100 C, 30 min + trypsin 60 min	—	—	—	—	—	—	—	—	—	—	—	+	—	—	+	—	—	—

TABLI 2

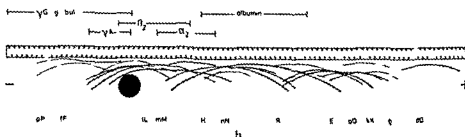
Frecipitinogens of the Reference Streptococcal Antigen Identified in Intracellular Materials from Streptococcal Strains Belonging to Different Types and Croups

Beta hemolytic streptococcus: Group	Type	Strain	Total number of precipitinogenic factors	Identified precipitinogenic factors: a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	r	s	t
A	12	I 13846 Reference strain	18	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	r	s	t
A	1	T 1-119 7	16	+	+	—	+	+	+	+	+	+	—	—	+	—	—	+	+	+	+
	3	S 84	16	+	+	—	+	+	+	+	+	+	?	+	+	—	+	+	+	+	+
	4	T 4 95—Rb5	17	+	+	—	+	+	+	+	+	+	—	+	+	—	?	+	+	+	+
	1?	8300	17	+	+	—	+	+	+	+	+	+	—	+	+	?	—	+	+	+	+
	25	B 346 op	16	+	+	—	+	+	+	+	+	+	?	+	+	?	—	+	+	+	+
	28	T 28 150A-4	18	+	+	—	+	+	+	+	+	+	?	+	+	?	—	+	+	+	+
	49	B 737 34 31	17	+	+	—	+	+	+	+	+	+	?	+	+	+	—	+	+	+	+
C	7	T 7 J	14	+	+	—	—	+	—	+	+	+	—	—	+	?	?	+	+	+	+
	20	T 0 (Neil)	15	+	+	—	+	+	—	+	+	+	?	+	+	—	—	+	+	?	+
		S P A K 39	9	—	—	—	?	+	—	—	+	—	—	—	+	—	—	+	+	?	?
		K 64 0-13	11	—	—	—	+	+	—	—	+	—	—	?	+	—	—	+	+	+	+
C	16	T 16 J	16	+	+	—	+	+	—	+	+	+	?	+	+	+	+	+	+	+	+
		F 68 A	10	—	—	—	+	+	—	—	—	—	—	+	+	—	—	+	+	+	+

Not primary human pathogenic streptccoccal strains



a



b

Fig 9

Photograph (a) and schematic drawing (b) of the immuno electrophoretic spectrum obtained with the reference streptococcal reactants The reference antigen IC A 1? (F 1384b) was applied in the circular basin and electroseparated the reference immune serum anti IC+FC was placed in the longitudinal basin The precipitate designations correlate with Fig 1

in two of the four group C strains The factor *h* was present in all A and C strains and the type 16 strain but could not be demonstrated in the other group G strain The analyses for the detection of the *o* factor were uncertain due to the faint precipitate formed by this antigen and its antibody The *m* factor was found in all group A strains with the exception of the type 1 and all group G strains but only in one of the four group C strains investigated All IC materials from strains showing the presence of the *m* factor had DPNase inhibiting capacity No antibodies were found in the anti IC+FC serum corresponding to the group A specific carbohydrate in the Lancefield extracts or the polyglycerophosphate preparation

Immuno Electrophoretic Analysis of the Reference Streptococcal Antigen

In order to further characterize the precipitinogens in the reference streptococcal antigen the reference antigen was separated electrophoretically in agar gel and analyzed with the reference immune serum Fig 2 a and b present the immune electrophoretic precipitation pattern which consisted of at least 19 precipitation arcs most of which were localized on the anodal side of the antigen reservoir Many of the reference precipitinogens were found on the anodal side of human serum

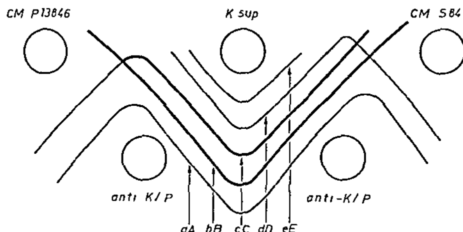


Fig 3
Schematic drawing of the precipitation pattern obtained when Lancefield extracts from a nephritogenic (CM – P 13846) and non nephritogenic (CM – S 84) streptococcal strain were analyzed with the kidney anti kidney immune system Precipitate designation is indicated

albumin By means of the comparative immune electrophoretic technique and employing heated and trypsinized preparations of IC materials from different strains as well as absorbed antisera twelve of the precipitation arcs could be identified with the precipitation bands in the aforementioned established reference precipitation spectrum These immune systems are labelled in Fig 2 b The identification of the other immune systems was not possible to perform due to the faintness of the precipitates or the multiplicity of precipitation arcs in certain regions Immune electrophoretic analyses performed at lower pH values (down to 5 8) than the one used in the standard technique did not permit further identification

Immunological Relationship between Streptococcal Cells and Human Kidney

Double diffusin in gel analyses of human kidney antigen (K sup) with anti human kidney serum absorbed with human plasma (designated anti K | P) revealed at least five separate immunoprecipitates These were designated *aA – eE* (Fig 3) When Lancefield extracts of the nephritogenic reference streptococcus (CM – P 13846) and a non nephritogenic streptococcus (CM – S 84) were analyzed with the same technique against anti K | P and compared with the reference kidney – anti kidney immune system two precipitates were formed with each of the Lancefield extracts and anti K P One of the two precipitates in each immune system coalesced with one immune precipitate of the kidney anti kidney system (*aA*) The other precipitate of the CM–C 84 – anti K | P system coalesced with the *dD* precipitate while the second

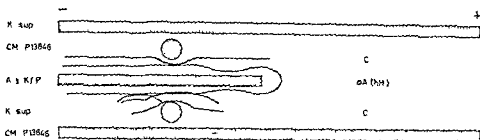


Fig 4

Schematic drawing of a combined comparative immuno electrophoretic analysis of the kidney antigens (K sup) and the Lancefield extract from the nephritogenic strain P 13846 using the anti kidney serum The precipitate designations correlate with Fig 3 serological relationship of the kidney aA and streptococcal hH precipitates is mentioned in the text

CM – P 13846 – anti K | P system coalesced with the cC precipitate of the kidney – anti kidney system No difference was noted in these precipitation patterns if an anti kidney serum was used which had previously been absorbed with human AB(+) erythrocytes

Comparative immune electrophoretic analyses of the kidney – anti kidney immune system and intracellular preparations and Lancefield extracts of cell walls from the two aforementioned streptococcal strains were also performed As may be seen in Fig 4 both the aA and the cC precipitates could be localized in the kidney – anti kidney immune spectrum By means of a particular basin arrangement it could also be illustrated that the aA precipitate of the reference streptococcal spectrum coalesced with the hH precipitate of the reference streptococcal spectrum but no antibodies against the other kidney antigens were demonstrable in the anti IC+FC serum Lancefield extracts of the other streptococcal strains containing the h factor developed a precipitate with the anti kidney serum which coalesced with the aA precipitate of the kidney – anti kidney system When the lung antigen was electroseparated and developed with anti K | P serum a precipitate was formed which coalesced with the aA precipitate of the kidney – anti kidney system

DISCUSSION

The multitude of precipitinogens in intracellular materials from streptococcal strains belonging to Lancefield's groups A C and G is illustrated by diffusion in gel methods is striking Although no attempts were made to analyze the chemical structure of these antigens the present results give enough information to make a tentative characterization of some of the precipitinogens in the reference intracellular streptococcal antigen preparation Thus it may be assumed that the q factor corresponded to the F₁ factor of Watson & Wiley (1963) because of its wide distribution among different A C and G streptococcal strains its heat and trypsin stability and its electrophoretic position The heat

TABLE 3

Summation of Results

Tentative Characterization of Streptococcal and Human Kidney Precipitinogens

Precipitinogenic factors	Properties
Reference streptococcal antigen	
a b g k	Related to human pathogenetic A C G strep strains
c	Specific to reference strep strain P 13846
f	Specific to strep Group A strains
h	Serologically related to human kidney antigen *a* and to a human lung antigen Common to strep Groups A C G
m	DPNase inhibitor Common to strep Groups A C G
n	Mucopeptide Common to strep Groups A C, G
q	E4 (*Wilson & Wiley* 1963) Common to strep Groups A C G
Human kidney antigen	
a	Serologically related to ref strep antigen *h* and a human lung antigen
c	Serologically related to a cell wall antigen from a nephritogenic strep strain (P 13846)
d	Serologically related to a cell wall antigen from a non nephritogenic strep strain (S 84)

and trypsin stable *n* factor might be correlated to the mucopeptide described by *Krause & McCarty* (1961) and which *Rotta et al* (1965) found could be released from streptococcal cell walls by ultrasonic treatment Presence of streptococcal mucopeptide in IC materials may offer an explanation to the excellent antibody response obtained in the sheep if considered in relation to a recent report on the adjuvant property of mucopeptides (*Holton & Schwab* 1966) According to Table 2 factors *a b g* and *k* were only found in beta hemolytic streptococci which are considered to be primary human pathogenic On the other hand the *a* and *b* factors were often difficult to demonstrate furthermore only three non human pathogenic strains were analyzed The characterization of these factors as typical for human pathogenic streptococci is therefore highly tentative and additional streptococcal strains should be analyzed The heat and trypsin labile factor *f* was only found in group A streptococci and might be regarded as a group A specific antigen not correlated to Lancefield s group specific carbohydrate as this latter substance resists trypsin treatment and 170 C The c and *f* factors were only found with certainty in the reference type 12 strain Both factors were heat labile and trypsin stable like the T substance of *Lancefield* (1954) Although the immunoelectrophoretic position of the *H* also corresponded to the electrophoretic position of

the T-substance from a type 17 streptococcus reported by *Wilson & Wiley* (1963) further analyses should be performed to establish the correlation between the T substance and the c or l factors. The c, r, s and t factors were found in all A, C and G streptococci tested. Whether these four factors are specific for the A, C and G streptococci or are also present in streptococci belonging to other groups cannot be interpreted from the present results. The trypsin stable d factor was found in most A, C and G streptococcal strains. Although the immuno electrophoretic position of the dD precipitation arc corresponded to the position of streptococcal polyglycerophosphate as described by *Wilson & Wiley* (1963), the d factor cannot be the polyglycerophosphate as antibodies to this antigen were not detected in the anti IC+LC serum. The h factor could be demonstrated in all of the investigated A and C strains and also in the T 16 strain of group C, but could not be demonstrated in the F 68 strain. The correlation between this factor and antigens derived from normal human kidney and lung will be discussed below. The m factor was found to be unevenly distributed among the A, C and G streptococcal strains. On the other hand, IC materials from all strains which contained this factor also revealed DPNase inhibitory capacity. The immuno electrophoretic position of the mM precipitation arc corresponded to the d precipitate which was earlier shown by *Holm & Kayser* (1966) to be correlated to the DPNase inhibitor and its antibody. Furthermore the results concerning heat and trypsin lability indicated that the mM precipitate might be attributed to the inhibitor anti inhibitor immune system. A summation of the characterization of these precipitinogens is presented in Table 3. It must be emphasized, however, that this is only a tentative characterization. Additional strains should be analyzed and purified antigens should be prepared in order to verify the trend of the present findings. In a forthcoming publication (*Holm*, to be published) a further biochemical characterization of some of these factors will be presented together with results concerning the antigenic relationship between these factors and well known extracellular streptococcal factors such as streptolysin O, DNAse B, proteinase, streptokinase, DPNase etc.

The results concerning the antigenic relationship between human kidney antigen and streptococcal products showed that both intracellular material and Lancefield extracts from cell walls from nephritogenic and non nephritogenic streptococcal strains belonging to the serologic groups A and C and the T 16 strain of group C all contained a heat and trypsin stable precipitinogen (the h factor) antigenically related to a human kidney precipitinogen. Furthermore, this antigen was found to be related to a precipitinogen derived from normal human lung tissue. Immunological cross reactions between lung and kidney preparations have been reported by several authors (e.g. *Mellors et al.* 1955, *Bjorklund* 1956 and *Kaplan* 1964) and have been assigned to antigens present in the capillary vascular bed. In 1958 *Kaplan* reported that rabbits in

the T substance from a type 17 streptococcus reported by *Wilson & Wiley* (1963) further analyses should be performed to establish the correlation between the T-substance and the *c* or *l* factors The *e*, *r*, *s* and *t* factors were found in all A, C and G streptococci tested Whether these four factors are specific for the A, C and G streptococci or are also present in streptococci belonging to other groups cannot be interpreted from the present results The trypsin stable *d* factor was found in most A, C and G streptococcal strains Although the immuno electrophoretic position of the *dD* precipitation arc corresponded to the position of streptococcal polyglycerophosphate as described by *Wilson & Wiley* (1963) the *d* factor cannot be the polyglycerophosphate as antibodies to this antigen were not detected in the anti IC+LC serum The *h* factor could be demonstrated in all of the investigated A and C strains and also in the T 16 strain of group C but could not be demonstrated in the F 68 strain The correlation between this factor and antigens derived from normal human kidney and lung will be discussed below The *m* factor was found to be unevenly distributed among the A, C and G streptococcal strains On the other hand IC materials from all strains which contained this factor also revealed DPNase inhibitory capacity The immuno electrophoretic position of the *mM* precipitation arc corresponded to the *il* precipitate which was earlier shown by *Holm & Kaijser* (1966) to be correlated to the DPNase inhibitor and its antibody Furthemore the results concerning heat and trypsin lability indicated that the *mM* precipitate might be attributed to the inhibitor anti inhibitor immune system A summation of the characterization of these precipitinogens is presented in Table 3 It must be emphasized however that this is only a tentative characterization Additional strains should be analyzed and purified antigens should be prepared in order to verify the trend of the present findings In a forthcoming publication (*Holm* to be published) a further biochemical characterization of some of these factors will be presented together with results concerning the antigenic relationship between these factors and well known extracellular streptococcal factors such as streptolysin O DNAse B proteinase streptokinase DPNase etc

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

7 *Adoptive Immunization of Virus Carrier Mice by Grafts of Normal Syngeneic Lymphoid Cells*

By

JØRGEN HANNOVER LARSEN and MOGENS VOLKERT

Received 11 x 66

Billingham et al (2) demonstrated in 1956 that normal syngeneic lymphoid cells are capable of abolishing a state of immunological tolerance when transplanted into tolerant animals This was later confirmed by the same group (3) and by *Gowans et al* (8) In the experiments described by these authors the state of tolerance was induced to allogeneic tissue and spleen cells thoracic duct cells and peripheral blood leucocytes were also employed *Volkert* (22) has shown that the same results could be obtained in another tolerance system he found that normal spleen cells were able to abolish the tolerant state in LCM virus carrier mice

As the role of the normal lymphoid cell is one of the fundamental problems in the question of tolerance the effect of these cells in LCM virus carrier mice was reinvestigated using varying cell doses and long observation time The effect of immune lymphoid cells has been described in our previous papers (21 22 23 24 25 28) The results of the normal cell experiments will be recorded in the following

MATERIAL AND METHODS

The tolerant LCM virus carr er m ce were of the highly inbred C3H st ain used in our previous experiments The toler n e was indu ed b neonatal injection of LCM virus Only female m were employed

The normal (unsen it ed) donors us d w e f om the ame inbred strain and had never been n conta t w th LCM iru

The preparation of ell u pen ions the transplant tion the se ological and viro l g al methods were the ame desc bed in our previous p pers (? 24 26 28)

The authors are nd bted to Mrs *B Højer Simonsen* for sk llful technical assi t ance

Th e Inst tute f Tumour Virus R ea ch is suppo ted by g ants fr m the *Einar Willumsen s Mem al Fun l* and the *Danish Can er So iety*

In the following experiments normal (unsensitized) lymphoid cells were taken from a pool of cells harvested from the spleens and lymph nodes of normal C3H female mice The cells were transplanted into syngeneic virus carriers in doses of 10×10^6 100×10^6 200×10^6 and 1000×10^6 After the transplantation blood samples were taken on days 10 20 30 40–45 60 100 140 190 and 220 In addition for the 10×10^6 group blood samples were taken on day 420 after the transplantation for the 100 and 200×10^6 groups on days 330 and 420 after the transplantation and for the 100×10^6 group on day 280 but not later than this All mice survived an observation period of 190 days After this time there were sporadic deaths of a few mice in each group As is apparent from the figures these deaths after the 190th day did not however influence the experimental results

The blood samples from each individual mouse were tested for virus and complement fixing antibodies The blood was taken from the inner canthus of the eye A small amount of heparin just enough to hinder coagulation was added to the blood for virus titration Shortly after the blood was taken serial decimal dilutions were made and each dilution was injected intracerebrally into four white Swiss mice The whole procedure was completed within an hour of the blood being taken No heparin was added to the blood for the complement fixation test these samples being allowed to coagulate The serum was then separated after centrifugation at 3000 rpm for 20 minutes inactivated on a water bath at 56 C for 30 minutes and stored at 4 C The complement fixation tests were carried out once a week

From the data obtained it was possible to distinguish five main types of experimental results (A B C D and E) in the whole material In Figs 1 to 5 these five types are shown graphically Each of the five pairs of curves indicates the course of the viremia and antibody titre in a single transplanted virus carrier mouse representative of the type The A curves (Fig 1) are from a mouse of the 10×10^6 group The B C- and D curves are shown in Figs 2 3 and 4 and are all from mice injected with 100×10^6 cells The E-curves (Fig 5) are from one in the 1000×10^6 group In each group minor deviations from the curves shown were seen but these will be described in the text

Control group A fifth group of 1[illegible] C3H female virus carrier mice was used as control group They were left untreated *i e* they were not transplanted The virus titres of the blood and the antibody titres were determined as described above for the other groups All the mice in this group had a course of the A type in Fig 1 At no time did the virus titre fall below 10[illegible] and the complement fixing titres were <4 These results are in accordance with previous findings in more than 100 untreated virus carrier C3H mice kept as controls in other kinds of experiments in this laboratory

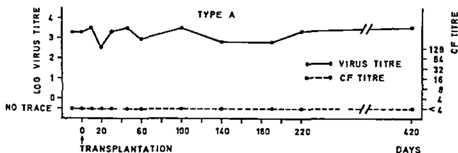


Fig 1

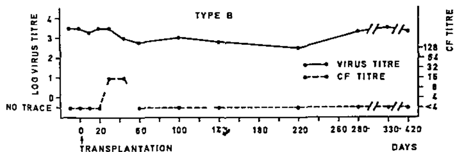


Fig 2

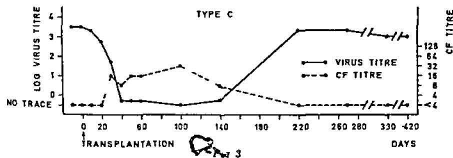


Fig 3

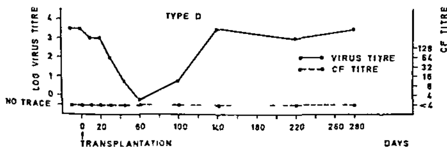


Fig 4

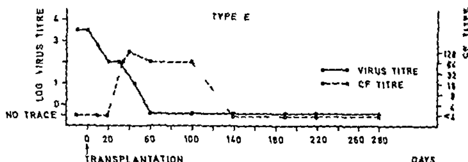


Fig 5

Figs 1–5

Blood virus titres and complement fixation antibody titres of tolerant LCM virus carrier mice following transplantations of lymphoid cells from unsensitized syngeneic donors Each figure indicates the data from one individual mouse representative of the group to which it belongs The mouse in Fig 1 has received 10×10⁶ cells those in Figs 2 to 4 have received 100×10⁶ cells and the mouse in Fig 5 1000×10⁶ cells

The effect of 10 × 10⁶ syngeneic lymphoid cells In the following experiment a dose of 10 × 10⁶ normal lymphoid cells isolated from the spleens and lymph nodes of syngeneic female donors was transplanted intraperitoneally to seven female C3H virus carriers The blood virus titres and the complement fixing antibodies were followed at intervals for the next 420 days The values for one of these mice are recorded graphically in Fig 1 All the seven mice had a course of the A type At no time did the virus titre of the blood fall below 10 and the antibody titres were <4 The course is identical with that of the untreated control group i e no measurable adoptive immunization took place in these transplanted animals

The effect of 100 × 10⁶ syngeneic lymphoid cells A group of 15 female C3H virus carriers was each transplanted intraperitoneally with a dose of 100 × 10⁶ normal syngeneic lymphoid cells isolated from the spleens and lymph nodes of normal female donors The blood virus titres and antibody titres were followed as described above

From the data obtained in the individual mice three types of results could be seen in this group In three mice the course was of the B type (Fig 2) In these mice no virus titre below 10 was found but complement fixing antibodies were produced in all of them during the first two months after the transplantation One month after the transplantation titres of about 16 were observed However the antibodies soon disappeared and did not reappear This means that a very short lived and weak adoptive immunization took place during the first two months after the transplantation as indicated by the presence of complement fixing antibodies It did not however influence the viremia

Eight further mice from this group had a course of the C type although the course in some mice differed slightly from the curves shown (Fig 3) In all these mice the blood virus titres decreased and

the minimum titres were reached two to three months after the transplantation In four mice the virus in the blood disappeared completely in the other four mice the titres fell to between $10^{1.3}$ and 10 [illegible] The minimum titres were found in some mice for only a short time in a few at only one observation whilst in other mice the minimum titres were maintained at a constantly low level for several months In all eight mice however the virus titres increased again and after one year all the titres were more than 10 [illegible] *i e* at the virus carrier level

In these eight mice complement fixing antibodies were formed in small to moderate amounts (titres of 16–64 max) during the first six months Then they disappeared and did not reappear In these mice a quite strong but temporary adoptive immunization took place as judged by both the temporary decrease of the viremia and by the presence of antibodies

The remaining four mice of the 100 × 10 group had a course of the D type (Fig 4) The minimum titres for these four mice were reached at 60 days after the transplantation and were of trace amounts in two mice and of titres of $10^{0.8}$ and 10 [illegible] in the other two In all four mice the blood virus titres hereafter returned to the virus carrier level The antibody titres for these four mice were all negative (<4) during the whole observation period This indicates that in these mice there was a temporary adoptive immunization as judged by the decrease in the blood virus titres but in spite of this no antibodies were formed

The results for the whole 100 × 10 group show that a few mice will develop a short lived and weak adoptive immunization characterized by low titre antibodies but not influencing the viremia In a few mice the induced immunization depresses the virus titres but does not cause antibody production The main part of the mice will however develop a quite strong but temporary adoptive immunization which as long as it lasts influences the virus titres and leads to production of antibodies

The effect of 200 × 10 syngeneic lymphoid cells Nine female C3H virus carriers were transplanted intraperitoneally with 200 × 10 lymphoid cells isolated from the spleens and lymph nodes of normal syngeneic female donors The viremia and the complement fixing antibodies were followed as described above

Two types of results were seen in this group Three mice showed a course of the C type (Fig 3) not differing essentially from that described in the foregoing section on 100 × 10 cells A temporary adoptive immunization took place Six mice had a course of the F type (Fig 5) The virus titres decreased to values ≦10 40 to 60 days after the transplantation and stayed on this low level for the remainder of the 410 day long observation period

Complement fixation and neutralization tests were in this group not carried out on individual samples but on a pool of serum from all members of the group The complement fixation antibody titres were demonstrable from the third week and during the remaining observa

tion period the titres varied from 64 to 128. The classification of this group is therefore made only according to the course of the viremia. The titres of the neutralizing antibodies in this group were followed for the first 70 days after the transplantation. These antibodies appeared in the second week after the transplantation, reached maximum values six weeks later with a log neutralization index of 3.0 and then decreased again. Seventy days after the transplantation the log index was 1.8. Further tests were not made.

The results from these six mice revealed a complete and long lasting abolition of the tolerant state as is seen after transplantation of a sufficient number of immune cells.

The effect of 1000 × 10⁶ lymphoid cells. A group of seven female C3H virus carriers was transplanted intraperitoneally with 1000 × 10⁶ lymphoid cells isolated from the spleens and lymph nodes of normal syngeneic female donors. The viremia and complement fixing antibodies were followed for the next 270 days.

From the course of the viremia and antibody titres in the individual mice it was possible to distinguish two types of response in this group. Four mice had a temporary adoptive immunization (type C, Fig. 3). The minimum titres of all four mice were below 10. In all these mice the virus titres again increased to virus carrier values (>10⁻⁵). Three mice showed, however, a permanent adoptive immunization (type E, Fig. 5). Minimum virus titres of no trace were reached two months after the transplantation and stayed at this low level for the remainder of the observation period (280 days).

Complement fixing antibodies appeared four weeks after the transplantation. In all mice of this group antibodies were present. They reached maximum titres (32–128) 40 days after the transplantation. During the following period they dropped slowly to low levels or disappeared.

Table 1 contains an outline of the four groups described here and also of the control group of non transplanted virus carriers.

TABLE 1

Survey of the Course of the Viremia in Female C3H Virus Carrier Mice Transplanted with Varying Numbers of Lymphoid Cells Isolated from the Spleens and Lymph Nodes of Normal Syngeneic Female Donors together with a Control Group of Non Transplanted Virus Carriers

Cell number transplanted	Number of mice in the group	Type A	Type B	Type C	Type D	Type E
10 × 10⁶	7	7	0	0	0	0
100 × 10⁶	15	0	3	8	4	0
500 × 10⁶	9	0	0	3	0	6
1000 × 10⁶	7	0	0	4	0	3
Control	15	15	0	0	0	0

DISCUSSION

It is generally believed that adoptive immunization of tolerant animals is brought about by a repopulation of the recipients with the living immunologically competent cells from histocompatible (= syngeneic) donors and by the immunity conferred by these cells However in accordance with the experimental evidence presented by *Mäkela & Mitchison* (14) and by *Celada* (5) the recipients seem to possess a barrier to the repopulation with transplanted syngeneic cells or/and to the function of these cells As the injected cells are compatible to their new host they should share the fate of the host s own cells It therefore seems logical to assume that this barrier is caused by the same mechanism which must be operative in checking the number and the function of the lymphoid cells in a normal mouse Unfortunately this mechanism is completely unknown but it has been suggested that there might be a sort of *competition between the lymphoid cells* This could be merely a question of Lebensraum but such phenomena as contact inhibition gamma globulin feedback humoral depression and other factors might also play a role

Where mice tolerant to the LCM virus are concerned previous experiments in this laboratory (23 24) have shown that to obtain a detectable adoptive immunization a minimum of 5–10 $\times$ 10 lymphoid cells from immunized donors has to be injected into the virus carrier mice The 5–10 $\times$ 10^6 cell doses are about 1/40 of all the cells contained in the spleen of an immunized donor If one can judge from results of experiments in which other antigens than the LCM have been used as immunizing antigens in mice (9 11) the 5–10$\times$ 10 cell dose mentioned above should contain about 10 to 10^3 sensitized cells The mean generation time for immunologically competent cells according to *Nossal & Makela* (15) and to other investigators (4 10 18) is about eight to twelve hours An uncontrolled growth of much smaller amounts of sensitized cells than those contained in the minimum active cell dose should therefore multiply in the course of a few days to give a huge amount of immunologically active cells and cause a clear-cut immune response The fact that such a reaction is not detectable when cell doses of less than 5 $\times$ 10 cells are injected strongly supports the hypothesis that a control mechanism in the host restricts the growth or the function of the injected cells

The effect of the control mechanism on sensitized cells seems however to depend on the number of cells injected and on the antigenic stimulation the cells receive When the cell dose is high enough and the antigenic stimulus is very strong the cells will not only survive and function but seem to be able to overcome the control mechanism completely

This phenomenon is apparent from the fact that a dose of about 100 $\times$ 10 lymphoid cells from immune donors i e about only one fifth

from our experiments that a kind of balance exists between cells and control mechanism When small cell numbers are injected the control mechanism completely suppresses the cells on the other hand in cases where large doses of sensitized cells are employed the cells might out balance the control mechanism The influence of cell number on the outcome of the balance might perhaps also play a role a long time after the cells have been transferred Perhaps in cases in which the population of cells never reaches a high level the balance might tip to the advantage of the control mechanism in the course of time and then the immunological activity and perhaps the proliferation and life of the cells will be brought to an end In cases where sensitized cells are employed this might never happen simply because these cells are highly susceptible to the antigenic stimulus they receive when injected into the virus carrier recipients As pointed out above this stimulus probably supports the colonization As a result a large cell population is likely to be produced in all cases in which the initial cell dose has been high enough to overcome the immediate effect of the control mechanism However normal cells are probably not stimulated to the same degree and judging from the antibody titres obtained and from the influence on the virus in the recipients the number of immunologically active cells derived from the injected normal cells will never reach a high level In such cases the control mechanism might have a chance to suppress the cells even after they have settled and the return of tolerance might be the result

The phenomenon of a change from tolerance to immunity and back to tolerance has also been observed under other experimental conditions with the LCM virus *Hannover Larsen* (12) has shown that this happens in infected infant mice if the infection is postponed until the end of the period in which mice are fully susceptible to the induction of tolerance *Hannover Larsen* found that babies infected at the age of four to nine days can develop tolerance to the virus but this tolerance is only temporary In the course of time antibodies will appear and this is associated with a drop in the blood virus titres However later a full degree of tolerance often reoccurs spontaneously These finding also point to the existence of a balance between immunologically reactive cells and control mechanism These results will be presented and discussed in a later paper

Chase (6) has reported that in adoptive immunization of guinea pigs with sensitized cells the ability to produce antibodies and the state of hypersensibility conferred by the same batch of cells did not run parallel in different animals In some of the recipients the ability to produce antibodies largely superseded the hypersensibility in others the reverse was the case *Chase* points out that these results indicate that the antibody production and the hypersensibility are caused by different cells Other investigators (1 7 16 17 19) have also obtained experimental evidence pointing in the same direction From our experi

ments it is apparent that the production of complement fixing antibodies and the decline of the blood virus titres similarly do not run parallel We have mice in which there is a demonstrable antibody production simultaneous with constant high blood virus titres and on the other hand we have mice in which the virus disappears in the blood in spite of the fact that no complement fixing antibodies can be detected In a previous paper (27) we have pointed out that we have many other data which indicate that the elimination of the virus in adoptively immunized virus carriers is most probably caused by a direct cellular function and not by the antibodies The results presented in this paper support this view but also seem to support the idea that different cells are responsible for the different parts of the very complex immune mechanism Moreover the effect of the control mechanism on these different kinds of cells seems to vary and to differ making an independent action of one or other group possible

SUMMARY

The effect of adoptive immunization with normal (unsensitized) syngeneic lymphoid cells on tolerant LCM virus carrier mice using varying cell doses has been investigated

10×10 cells gave no measurable adoptive immunization 100×10 cells gave a weak and short lived adoptive immunization in some mice in others a stronger but still temporary adoptive immunization 200×10^6 and 1000×10 cells gave a quite strong adoptive immunization in all mice but led to a long lasting abolition of the tolerant state in only some of the mice

In many mice there is no parallelism between the two measured functions of the adoptive immunization the antibody formation and the virus elimination This indicates that two functionally different systems of immunologically reactive cells are involved

The problem of cell dose with particular regard to a homeostatic cell control mechanism of the animal is discussed

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STUDIES ON THE CELL TOXICITY AND SPECIES SPECIFICITY OF PURIFIED STAPHYLOCOCCAL TOXINS

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In the efforts to understand the pathogenesis of staphylococcal infections many approaches have been tried. Several papers offer evidence which strongly suggests that the staphylococci owe at least part of their pathogenic capacity to extracellular toxins and enzymes (6, 24). Factors which exert cytotoxic action on human cells are of course of special interest.

To study this problem many authors have used cell cultures as experimental models. Most works concern alpha hemolysin which has been reported to have a cytotoxic effect on various cell species, such as rabbit smooth muscle and kidney cells (1, 7, 23), mouse kidney cells (7), chick embryo cells (21), guinea pig skin (17), human amnion, kidney and skin cells (1, 7, 17) as well as Ehrlich ascites carcinoma cells (18).

In some reports the cytotoxic effect of other toxins has also been investigated. *Korbecki et al.* (16) found strong cytotoxic activity by both alpha- and beta-hemolysins on KB and monkey kidney cells. Deoxyribonuclease had feeble activity on KB cells and stronger on H.Ep.2 cells. Neither leucocidin nor staphylokinase were cytotoxic for the cell cultures studied.

According to some authors, no cytotoxic effect was observed when purified enterotoxin B preparations were tested on H.Ep.2, HeLa and human heart cells (20) or monkey kidney cells (14). This disagreed with the findings of other authors. *Guerin et al.* (9) found a cytopathogenic effect on chick embryonic heart fibroblast and *Schaeffer et al.* (22) a cytotoxic effect on cell cultures of human embryonic intestine.

The toxin preparations used are, however, often crude or unsatisfactorily characterized. Furthermore, earlier reports have not systematically dealt with the effect of various toxins on human cells.

In this report the effect with regard to cell toxicity and species specificity was followed in fractionation experiments whose primary aim was the study of factors toxic to human cells. The results were

also considered in relation to the effect on other cells The purity of all preparations was immunologically characterized As alpha beta and delta hemolysin in this work were tested on cells other than erythrocytes they were named alpha beta and delta lysin resp

MATERIALS AND METHODS

Strains Staphylococcus aureus strains SC and 196E (3) were used for toxinproduction

Preparation of crude toxin for fractionation Crude toxin was produced on solid medium covered with cellophane as described earlier (11) The pooled extracts were concentrated according to *Kohn* (15)

Toxin assay Coagulase enterotoxin B leucocidin hyaluronidase lipase alkaline phosphatase staphylokinase and DNAase were determined as described in previous papers (10 11)

Enterotoxin A assay 1) *In vitro* The preparations were tested for enterotoxin A in gel diffusion (25) with a purified antienterotoxin A serum kindly supplied by Dr *F P Casman* 2) *in vivo* The cat test was used as described in a previous paper (12) except that the preparations were not boiled before the injection but neutralized with a serum containing high anti alpha and anti beta lysin titres (Burroughs Wellcome l t no RA 388)

Proteolytic enzyme Plates for testing proteolytic enzyme activity were prepared using matrices for basin plate technique according to *Ouchterlony* The agar (? per cent) contained 1 per cent gelatine The basins were filled with the samples and the plates were incubated at 37 After 12–18 hours the plates were flooded with saturated ammonium sulphate

In the presence of proteolytic enzyme activity a clear zone appeared around the basin

Alpha beta and delta lysins were determined as described earlier (10) except that all dilutions for beta lysin were performed in phosphate buffered saline (pH 7 0) containing 10 3 M Mg SO_4 according to *Haque et al* (13) So rabbit sheep and human erythrocytes were used for the titration of alpha beta and delta lysins resp

All titrations were read both after 1 hour at 37 and after 1 hour at 4

Cell Cultures A calf serum adapted strain of HeLa cells originally adapted to horse serum according to *Mandel* (19) was grown in Hank s salt solution enforced with 0 1 per cent yeast extract and 0 1 per cent lactalbumin hydrolysate and containing 20 per cent calf serum KB cells were grown in the same medium

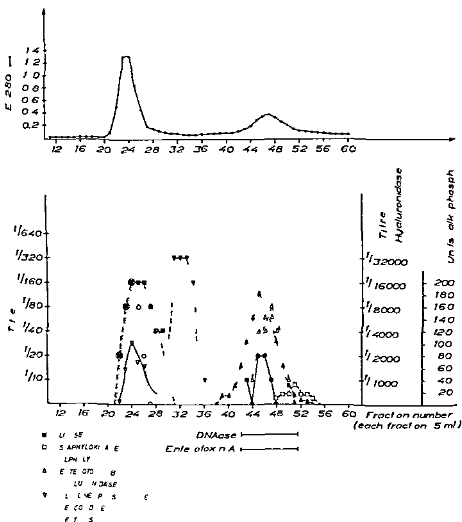
Primary human kidney cells and foetal colon cells were grown in Eagle s medium (?) with 10 per cent calf serum Primary cultures of calf cells were prepared from trypsinized kidneys of newborn calves and grown in Hank s solution with 0 5 per cent lactalbumin hydrolysate and 10 per cent calf serum

Primary cultures of cynomolgus monkey kidney cells were grown in Eagle s medium with 6 per cent calf serum After outgrowth the cultures were changed to Eagle s medium without serum The cells were grown in plastic petri dishes at 37 in a humidified atmosphere of 5 per cent CO in air All media contained penicillin (100 IU/ml) and streptomycin (100 µg/ml)

Granulocytes adherent to cover glasses were prepared according to *Cristofine* (8)

Cytotoxic studies Before use all toxins were calibrated to 0 02 M sodium phosphate buffer containing 0 1 M NaCl pH 7 2 7 4

Primary assay of cell toxicity Plate culture with the cells to be tested were overlaid with five ml agar containing 0 17 per cent $NaHCO_3$ 4 per cent calf serum and 0 95 per cent special agar Noble (Difco) in Eagle s medium When the agar had solidified it was cut with an agar gel cutter and the agar core removed by gentle suction The resulting wells were then filled with the toxin material which was serially diluted in Eagle s medium After incubation at 37 in 5 per cent CO in air for the intended time usually 2 hours the toxin was removed by suction and the plates covered with 5 ml of the same agar overlay containing neutral red at a concentration of 1/20000 The plates were usually read after 6 and 16 hours Cell death was roughly indicated by loss of ability to stain with neutral red



F g 1

Gel filtration of 10 ml crud toxin from stra n S6 th ou,h Sephadex G–100 Th extinction at 280 mμ as well as the localization and titres f different activities are indicated D\Aa e and ent t xin A present in er low tit es are indicated below

Radio sotope stu (s Tt totox c effect vas also studied b follow n, the release of P3 previously n orp r ted int plat ell ultures The plate were w shed nce with phosphate buffe d l ne (PBS) and (1 mC of carrier f ee sodium orthopho phate (Radiochem c l (ent Amer h m England) in 5 ml pho phate free Fagle medium added Aft r 4 h ur the medium as ucked ff the plat wa hed once with PBS and 5 ml Eagl m dium dd d Aft r a furthe 24 hours the m dium was removed the cell wa hed 4 t mes with PI S nd th tox n to be tested appr priatel diluted in F gle m dium added t the ll n 5 ml 10 tions S mples f 0 1 ml were withdrawn at a iou interval nd the adioactivity counted in a thin end window Geiger t e

Immuni ation) e lu and in u electrophoresis was performed as d bed earlier (12)

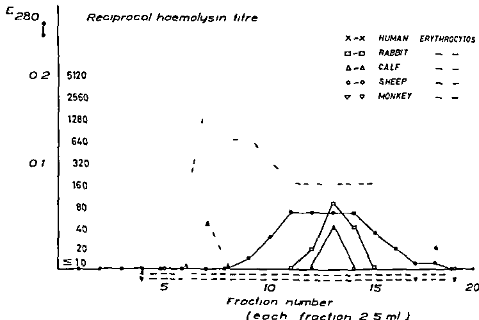


Fig 2

Preparative electrophoresis of a 196F alpha lysin peak from gel filtration on a Sephadex (– 5 c lumn void volume 6 ml Two ml of the material was run towards the cathode at 500 V for 24 hours in 0 05 M barbital buffer pH 8 2 The hemolytic effect on human rabbit calf sheep and monkey erythrocytes is indicated Broken lines indicate hot cold phenomenon

Gel filtration was performed on Sephadex G–100 (Pharmacia Fine Chemicals Uppsala Sweden) in a glass column 48 × 3 5 cm The elution buffer was 0 0? M NaI B containing 0 1 M NaCl pH 7 ? Elution was carried out at 4 at 1 5 ml/cm / hour and the effluent was collected in fractions of 5 ml

As shown earlier (10) Sephadex (–100 separates crude S6 toxin into three main peaks as is illustrated in more detail in Fig 1 Crude toxin from strain 1?F1 was fractionated in a similar way Beta lysin and proteolytic enzyme which were produced by strain 196F but not by strain S6 appeared in the third peak and enterotoxin A which was produced only in small concentrations by strain S6

Preparative electrophoresis was performed on a vertical column (50 2? cm) packed with Sephadex C 25 (Pharmacia Fine Chemicals) void volume (ml The column was cooled to 4 with a cooling jacket The fraction volume was ml Barbital buffer 0 05 M pH 8 2 was used

Preparative electrophoresis was used for further purification of the gel filtered materials Active material was always pooled concentrated (15) and dialyzed before the electrophoresis

Protein concentration was estimated routinely by measuring the absorption at 280 mμ in a Beckman DU spectrophotometer

Toxin and Enzyme Preparations

alpha lysin Two preparations of alpha lysin were tested 1) The first was the fraction from the Sephadex C 100 eluate of the strain S6 showing the highest titre As is evident from Fig 1 this material was contaminated with leucocidin enterotoxin B DNAase and staphyl kinase 2) The second was a 19?1 alpha l in peak from gel filtration further purified by preparative electrophoresis as shown in Fig 2 By this procedure the alpha lysin was partially separated from beta lysin Beside beta lysin small amounts of enterotoxin A could be shown by the cat test No proteolytic enzyme or leucocidin activity was found

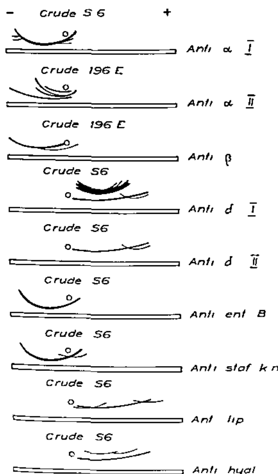


Fig 3
Schemati rej resentation of immunoelectrc phoretic ana l is of immuno era prepare l with purified t xinj rej ara ti ns Anti α I represents S6 material from econd peal f Sephadix C 100 anti α II 196E material after purifica tion by Sephadex G-100 and prej arative electroj horesi Anti δ I represent S6 material frm first j eak of Sephadex C-100 and δ II S6 material after j urification by Sephadex G-100 and preparative electrc phoresi I recipitation arcs present in preimmunesera are not illustrated in this fig

An immunoele troj horetical anal s of the tv o prepa at ns is given in Fig 3

beta lysin This tox n s t ted a materi l from the econd peak f gel filtration of the strain 196E purified by el trophoresis No ther acti it wa found in the preparation though two j re ipitation rcs developed hen crude 196F toxin was developed by anti beta l s n a shown in Fig 3

Delta lysin wa tested a 1) the top fraction from th fir t p al after gel f ltration on Sephadex C-100 of toxin from the strain SC or 196E) delta l in f strain S6 purified from l pase and alkalin phosphatase in j repa ti e el ctropho e i The material w s run toward th anode at 500 V f 4 h ur in 0 0 M ba bital buffe pH 8 9 A deta led tudy on the pu f cation f delta l s n a well as on the antig nic prope ties will be publish d ej aratel An mmun l ctroph ret al anal s of the two j reparations is given in Fig 3

Lipase The fract on d vas the fi t g l f lt ati n peak of the train S6 further purified by j reparati c l t ph re is a d ribed nder d lta l s n The j ified product was not perf tl parated frcm nt g n j resent n the delta lysin frac ti ns as is shown in Fig 3

Hyaluronidase v as t ken from the el filtr t n exp riments of stra n S6 The material conta ned ant g n j resent n b th lipa e nd delta l n prep r t on (Fig 3)

Enterotoxin B Immun l g ally pure enterotox n B was p pared b means f g l f ltrati n and prej t l tr ph r is a has b n j ubl h d sej arat ly (12)

Enterotoxin A has as yet not been adequately purified.

Staphylokinase was further purified from gel filtration (Fig. 1) by preparative electrophoresis towards the cathode at 500 V for 24 hours in 0.05 M barbital buffer pH 8.[illegible]. After this procedure the enzyme was present only in titres of 1/4. The preparations did not contain any alpha lysin or leucocidin. A small amount of enterotoxin B was however present (Fig. 3).

Alkaline phosphatase, DNAase, proteolytic enzyme and leucocidin: electrophoresis destroyed the activity of these enzymes preventing further purification.

EXPERIMENTS AND RESULTS

Effect of Crude Toxin

The toxin enzyme spectra of the free supernatants of the two strains S6 and 196E are shown in Table 1. Although these strains are both coagulase positive in slide and tube tests according to *Duthie* (4) no coagulase activity could be found.

TABLE 1

Toxin Enzyme Spectra of Staph. aur. *Strains S6 and 196E*

Toxin tested	S6	196E
Coagulase	—	—
enterotoxin A	+	+
enterotoxin B	+	—
alpha lysin	+	+
beta lysin	—	+
delta lysin	+	+
leucocidin	+	—
hyaluronidase	+	+
lipase	+	—
alkaline phosphatase	+	+
proteolytic enzyme	—	+
DNAase	+	—
staphylokinase	+	—

TABLE 2

Effect of Crude Toxin from Strains S6 and 196E on Different Cell Types

Celltype	S6	196E
HeLa cells	1/20	1/40
Monkey kidney cells	1/5	1/[illegible]0
Calf kidney cells	1/80	1/160
FB cells	1/5	1/10
Human erythrocytes	1/[illegible]0	1/160
Monkey erythrocytes	1/40	1/40
Calf erythrocytes	1/1[illegible]0	1/1280
Rabbit erythrocytes	1/1280	1/2560
Sheep erythrocytes	1/80	1/5120 (1/40 000)

Where the titres rose more than 2 steps after 1 hour at +4° C these values are given in brackets in addition to titres after 1 hour at 37° C.

The cytotoxic effects of the crude preparations were tested on erythrocytes and cells from various mammalian species as shown in Table 1. As this table shows both strains exerted a toxic effect on the cells tested. Higher titres were obtained with blood cells than with cell cultures from corresponding species. Further the effect on blood cells from various animals varied considerably.

Effects of purified toxins. Parallel to the purification procedure all fractions were tested on erythrocytes from man, monkey, calf, sheep and rabbit, on granulocytes from man as well as on human, monkey and calf kidney cells. Fractions with hemolytic or cytotoxic effect in the primary assay were further investigated in radioisotope studies on human, monkey and calf kidney and sometimes other cells.

Alpha lysin. As shown in Table 3 none of the preparations had any effect on red blood cells from man and monkey but elicited a strong action on calf and rabbit erythrocytes. The effect of alpha lysin of the strain 196E on sheep erythrocytes had hot cold character and was probably due to beta lysin present. No effect on human granulocytes could be demonstrated with this preparation. Alpha lysin from the strain S6 which lacked beta lysin had no or a very slight effect on sheep erythrocytes but attacked human granulocytes in a special way without lysis of the cell but with nuclear swelling and nuclear lysis. The table also shows that alpha lysin had a toxic effect on calf kidney cells but did not affect human and monkey kidney cells under the conditions studied. The effect of alpha lysin on tissue culture cells was also studied in repeated experiments by following the release of P^{3} from cells. The results of one such experiment are given in Fig. 4 which shows that alpha lysin had a greater effect on calf kidney cells than on human kidney cells. When cruder alpha lysin preparations were used human kidney cells were also attacked. The effects of alpha lysin on KB and HeLa cells and monkey kidney cells were usually less marked than on calf cells.

TABLE 3
Effect of α Lysin Preparations on Erythrocytes and Tissue Culture Cells from Various Species

Cell type	α lysin from S6	α lysin from 196E
Human erythrocytes	<1/10	<1/10
Monkey erythrocytes	<1/10	<1/10
Calf erythrocytes	1/40	1/40
Rabbit erythrocytes	1/3[illegible]0	1/80
Sheep erythrocytes	1/20	<1/10 – 1/160
Human kidney cells	<1/[illegible]	<1/[illegible]
Monkey kidney cells	<1/[illegible]	<1/2
Calf kidney cells	1/8	1/4

When two titres are given hot cold phenomenon is indicated

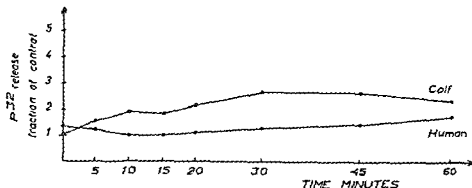


Fig. 4

Effect of alpha lysin on human and calf cells. Cells were labelled with P^{32} as described in Material and Methods. The hemolytic titre of the inoculated alpha lysin preparation was 1/64. Results are given as fraction of the control cultures from the same time.

Beta lysin. Beta lysin is characterized by so called hot cold hemolysis (6). As shown in Table 4 this toxin acted mainly on sheep erythrocytes and had no effect on red blood cells from man, monkey or rabbit or on human granulocytes. A weak effect was noted on calf blood cells. The preliminary tissue culture studies showed that beta lysin with a hemolytic titre of 1/2[illegible] had no visible cytotoxic effect on human, monkey or calf kidney cells. In the radioisotope studies beta lysin with a hemolytic titre of 1/2[illegible] had no effect on human and monkey kidney or KB cells and only a very slight effect on calf kidney and Mandel cells.

TABLE 4

Effects of β Lysin on Erythrocytes and Tissue Culture Cells

Cell type	37°	+37° and +4° C
Human erythrocyte	<1/4	<1/4
Monkey erythrocyte	<1/4	<1/4
Calf erythrocyte	<1/4	[illegible]
Rabbit erythrocyte	<1/4	<1/4
Sheep erythrocytes	<1/20	[illegible]
Human kidney cells	<1/2	n.t.
Monkey kidney cells	<1/2	n.t.
Calf kidney cells	[illegible]	n.t.

n.t. = not tested

Delta lysin (= leucolysin). As Table [illegible] shows delta lysin had a strong effect on human, monkey and rabbit erythrocytes and a weak one on calf and sheep erythrocytes. Corresponding hemolytic effect on erythrocytes from the various species could also be demonstrated with delta lysin after additional purification in preparative electrophoresis. Human granulocytes were quickly lysed after exposure to delta lysin. Of the tissue culture cells only human and monkey kidney cells were *affected*

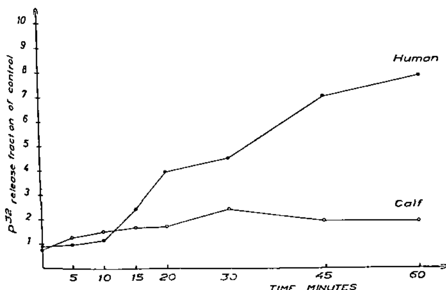
TABLE 5

Effect of δ Lysin on Erythrocytes and Tissue Culture Cells

Cell type	δ lysin from Sb	δ lysin from 196F
Human erythrocytes	1/80	1/40
Monkey erythrocytes	1/160	1/20
Calf erythrocytes	1/20	<1/20
Rabbit erythrocytes	1/80	1/20
Sheep erythrocytes	1/10	<1/20
Human kidney cells	1/4	1/4
Monkey kidney cells	1/2	<1/2
Calf kidney cells	<1/2	<1/2

as demonstrated by vital staining Delta lysin was also studied with the isotope method and one such experiment is shown in Fig 5 The results clearly show that delta lysin had a pronounced effect on the release of P^{32} from human kidney cells whereas the effect on calf kidney cells was weak The release of P^{32} from monkey kidney cells lay between that of human and calf kidney cells and was weaker than that with comparable titres of alpha lysin

Lipase The tested material which had a lipase titre of 1/10 had no



F j

Effect of delta lysin on human nd alf ells C lls e e labelled with P^{32} as described in Materi l and Meth ds The hemol t c titre of the inoculated delta l s n p epartion w 1/16 R ults ar given as fraction of th c ntrol culture from the ame t m

visible effect on erythrocytes or tissue culture cells from the specie used in this study.

Hyaluronidase. No visible effect was found on erythrocytes or tissue culture cells when hyaluronidase was used in a titre of about 1/20000. A slight effect was found by isotope studies with titres of 1/1280.

Enterotoxin B. Enterotoxin B had no visible effect on erythrocytes and tissue culture cells. Tissue culture cells were also tested by the isotope method with enterotoxin B preparation in a titre of 1/32 but no increase in the release of P^{32} was observed. An attempt to make tissue cultures from human embryonic colon resulted in growth of fibroblasts which were not injured by enterotoxin B.

Staphylokinase present in titres of 1/4 after gel filtration and preparative electrophoresis gave no effect on the cells tested. This was true also for the isotope studies where the material, however, was diluted further four times.

DISCUSSION

This report describes the effect of alpha-, beta- and delta-lysin, lipase, hyaluronidase, enterotoxin B and staphylokinase prepared to different degrees of purity on cells from various species. One of the strains S6 and 1961, depending on capacity, was used for toxin production. Alkaline phosphatase, DNA-ase, proteolytic enzyme and leucocidin which were produced by both strains were not studied in detail because their activity was lost during the purification procedure. Enterotoxin A has so far not been prepared free from alpha- and beta-lysin. Though both strains were coagulase positive no free coagulase could be found in the supernatants. The reason for this is unclear.

In order to reveal impurities all purified preparations were used for immunization and the sera were analyzed against crude toxin in immunoelectrophoresis.

Only enterotoxin B was purified to a state of immunological purity of the lysins beta- and delta-lysin were highly purified whereas alpha-lysin was purified to a lesser degree. This is further substantiated by the presence of other active enzymes in these preparations (Figs. 1 and 2). Staphylokinase was contaminated with enterotoxin B, lipase with antigens present in the delta-lysin preparations and hyaluronidase with antigens present in both lipase and delta-lysin preparations.

All fractions were tested on red blood cells from five species and tissue culture cells from at least three species. The effect on human granulocytes was also tested. The species specificity of the different fractions is partly defined since human erythrocytes are used for the titration of delta-lysin, rabbit erythrocytes for alpha-lysin and sheep erythrocytes for beta-lysin. The cytotoxic effect, as indicated by lack of vital staining after a two hour exposure of tissue cultures to toxin turned out to be rather insensitive with titres mostly 20–40 times

lower than those obtained in the corresponding erythrocyte tests The changes were also difficult to interpret in some cases The isotope method was found to be far more sensitive Human erythrocytes were attacked only by delta lysin and it was found that lipase and alkaline phosphatase had no effect

Human granulocytes were injured by delta lysin and also by a fraction from strain S6 containing alpha lysin leucocidin enterotoxin B DNAase and staphylokinase However the same cytopathogenic difference was noticed between these two preparations as the one observed by *Gladstone* between delta lysin and leucocidin (8) As enterotoxin B DNAase and staphylokinase were found to be noncytotoxic and human leucocytes are known to be very resistant to alpha lysin (2) the effect of the latter preparation was possibly due to leucocidin Because of the difference in cytotoxic picture no titres were given for human granulocytes

Human kidney cells were attacked by the delta lysin fractions and to a less degree by preparations from the second gel filtration peak of strain S6 containing chiefly alpha lysin They were very slightly influenced by purified alpha or beta lysins from 196F in comparable titres This difference between the two alpha lysin preparations is unclear As leucocidin as well as alpha lysin of strain S6 were lost when purified by preparative electrophoresis the effect could not be studied further but leucocidin was shown by *Korbecki et al* (16) to have no effect on KB H Ep 2 or L cells The feeble effect of hyaluronidase was probably due to contamination with delta lysin

In other reports alpha lysin has been shown to be cytotoxic for human skin explants (17) human amnion cell culture (18) KB cells (16) The experiments however vary considerably with regard to time of exposure and purity of the toxins and a comparison is therefore difficult The results in this paper support the opinion that enterotoxin B is noncytopathic to human cells (20) although *Schaeffer et al* (22) describes a cytotoxic effect after two days exposure of human embryonic intestine in tissue culture

Our findings with staphylokinase agree with those of other workers (16)

HeLa cells seemed to be more sensitive than human kidney cells in our experiments as it was strongly affected not only by delta lysin but also by alpha lysin and although slightly by beta lysin This shows the importance of the choice of cell line

KB cells were not injured by beta lysin in these experiments which disagrees with the finding of *Korbecki et al* (16) who however exposed their cells for 48 hours

Of cells from other species monkey and calf kidney cells were attacked both by delta and alpha lysin This was however not in accordance with the results on blood cells since monkey erythrocytes were not hemolyzed by alpha lysin Calf kidney cells were also damaged

by beta lysin The same was true for calf erythrocytes which were lysed in the typical hot cold manner though to a much less degree than sheep erythrocytes

Of all toxins tested the most interesting results concern delta lysin It is obvious that this toxin is active against cells from a wide range of species With respect to human cells this effect was however more pronounced than the effect of other toxins tested It might be that delta lysin has been neglected as a potential virulence factor of staph aur in human disease

SUMMARY

The celltoxic effect of partly purified staphylococcal alpha lysin beta lysin delta lysin lipase hyaluronidase enterotoxin B and staphylokinase was studied in short term experiments on erythrocytes and cell lines from different species as well as on human granulocytes Delta lysin was shown to exert a strong toxic action on human erythrocytes human granulocytes and human kidney cells The effect of alpha lysin on the same cells was much less pronounced On the other hand alpha lysin exerted a stronger effect on calf cells than delta lysin The other toxins and enzymes studied could not with any certainty be correlated to any cytopathogenic effect at 37

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ANTIGENIC STUDIES ON *MICROCOCCUS* STRAINS

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The taxonomic position of the micrococci is still very confusing. Two main features seem, however, to have been accepted by recent authors. First, micrococci can most reliably be distinguished from staphylococci by their inability to attack glucose anaerobically (2, 5, 6, 19). The validity of this distinction has lately been supported by investigations on the DNA base composition of members of the two genera (4, 1). Second, the genus *Micrococcus* can be subdivided into two groups based upon the ability or inability to attack glucose aerobically (15, 5).

Apart from this subdivision, there is little agreement on the classification of micrococci into species or groups, of which a large number have been described. In recent years *Kocur & Martinec* (15) have recommended that the genus *Micrococcus* should have only 6 species and 2 varieties. *Baird-Parker* first recognized 7 subgroups (2), later 8 (3). *Evans* (6), like *Bohacek et al.* (4), is of the opinion that at present only two species, i.e. *M. luteus* and *M. roseus*, should be recognized, as others lack the homogeneity that should characterize valid species.

The antigens of the members of the genus *Micrococcus* are unknown. A study of micrococcal antigens, including their comparison with staphylococcal antigens, which are relatively well known, might contribute to the understanding of the micrococci. The present investigation has been carried out on a selected but limited material of culture collection strains classified as micrococci.

MATERIAL AND METHODS

Strains

Twenty *Micrococcus* strains were obtained from the Czechoslovak Collection of Microorganisms through the courtesy of Dr. *M. Kocur*. The strains had been selected so as to represent differences in biochemical activities and at the same time to represent some of the formerly recognized species. They had been classified according to the scheme of *Kocur & Martinec* (15). The material consisted of:

- 7 strains of *M. luteus* (CCM 810, 144, 1335, 210, 857, 686, 310)
- 5 strains of *M. roseus* (CCM 146, 691, 606, 861, 142)
- 4 strains of *M. conglomeratus* (CCM 740, 547, 84, 757)
- 4 strains of *M. varians* (CCM 529, 884, 719, 1176)

The strains were examined for colony and pigment formation on blood agar plates, cell morphology in Gram preparations from blood agar cultures, production

of catalase and coagulase acid production from glucose aerobically and anaerobically and typability with *Staph aureus* phages None of the strains produced coagulase or was typable by phage All produced catalase Two strains (*M conglomeratus* 757 *M varians* 599) undoubtedly produced acid from glucose anaerobically (19) None of the *M luteus* or *M roseus* strains attacked glucose aerobically whereas all the *M conglomeratus* and all the *M varians* strains except *M varians* 719 did so

The two strains which attacked glucose anaerobically could not therefore be accepted as micrococci but were probably staphylococci The third irregular strain appeared to belong to another *Micrococcus* species than that in which it had been placed These three strains are included in the antigenic study for comparison A number of control strains of *Staph aureus* and *Staph epidermidis* have also been examined

Methods

All the *Micrococcus* strains as well as the control strains were examined in *Micrococcus Staph aureus* and *Staph epidermidis* immune sera by slide agglutination ring test and agar gel precipitation and haemagglutination of sensitized normal and tanned sheep erythrocytes

Immune sera were obtained by intravenous injections of formalin killed bacteria in rabbits (16) Factor sera were produced according to the method of *Oeding* *Agglutination* was performed on slides using live bacteria For technical details see *Haukenes* (10) and *Hofstad* (12)

Ring test precipitation was carried out with the supernates of saline extracts of the bacteria against undiluted immune sera The *agar gel diffusion* technique was as described in (11) Eighteen hr live nutrient agar cultures of the strains were tested against undiluted whole sera Polysaccharide A (9) polysaccharide 263 (13) protein A (7) and antigen D (14) from *Staph aureus* and the wall teichoic acid from *Staph epidermidis* strain T 2 and from *Micrococcus* strain I 3 (18) were included as references

Haemagglutination Normal and tanned sheep erythrocytes were sensitized with saline extracts of the bacteria and agglutinated in *Staph aureus* Cowan I immune serum which contains antibodies against *Staph aureus* sensitizing antigens (17)

RESULTS

Agglutination

Agglutinins in rabbit preimmune sera A number of rabbit preimmune sera were examined for the presence of agglutinins against micrococci Table I illustrates the reactions in 3 sera The majority of the *Micrococcus* strains agglutinated strongly in undiluted preimmune sera Most were negative in 1/10 dilution but some reactions were observed up to 1/40–1/80 *Staph aureus* and *Staph epidermidis* gave similar results

TABLE I

Agglutination in Rabbit Preimmune Sera

	No of strains tested	No of strains agglutinating in											
		Serum I				Serum II				Serum III			
		1/1	1/10	1/20	1/40	1/1	1/10	1/20	1/40	1/1	1/10	1/20	1/40
Micrococcus	18	15		1	0	16	7	2	1	1	2	1	0
Staph aureus	19	11	4	4	0	14	4	2	1	8	0	0	0
Staph epidermidis	8	7	3	1	0	5	1	0	0		0	0	0

TABLE 2

Absorption of Rabbit Preimmune Serum II

Strains agglutinated		Serum titres				
		Before absorption	After absorption with			
			M luteus 810	*M conglom* 84	*Staph aur* 830	*Staph epid* 1622
M luteus	810	80		40		1
M conglom	84	10	10	–		
Staph aur	830	40	40	20		20
Staph epid	1622	20	20	1	1	–

Reciprocal values
– means no agglutination in undiluted serum

Absorptions were carried out to see if all reactions were due to one and the same antibody Table 2 shows that this cannot be the case The agglutinations observed in preimmune sera are produced by more than one antibody probably several some of which are apparently common to micrococci and staphylococci whereas others are more specific A micrococcal immune serum which did not agglutinate the homologous strain prior to immunization may therefore nevertheless contain preimmune antibodies in low titres against other micrococcal strains and against staphylococcal strains This can hardly be avoided Preimmune serum had not been preserved in sufficient quantities to permit control agglutinations with all strains which were positive in the corresponding immune serum By agglutination in immune serum it was therefore difficult to evaluate the low titres which might be due either to preimmune or to immune antibodies Titres ≧ 1/40 in the following are estimated as significant for immune antibodies

Immune sera against Micrococcus Staph aureus and Staph epidermidis strains cross agglutinations and cross absorptions The Micrococcus strains and a number of Staph aureus and Staph epidermidis strains agglutinated in Micrococcus Staph aureus and Staph epidermidis immune sera The reaction pattern was the same as in preimmune sera i e extensive cross reactions in low titres The production by immunization of genus heterologous antibodies did not seem to be very extensive but could easily be demonstrated particularly in immune sera which had only small amounts of preimmune antibodies Table 3 gives an example of this Cross reacting antibodies are therefore both preimmune and immune apparently depending upon which strains of staphylococci and micrococci the rabbits have experienced before immunization

Successive absorptions were then carried out in 10 Micrococcus immune sera The sera were agglutinated before absorption and after each absorption with the set of strains mentioned above The absorptions confirmed that micrococci and staphylococci have more common

antigens When micrococcal immune sera were absorbed with one or two *Staph aureus* strains agglutination regularly disappeared with all staphylococcal strains but also with the majority of *Micrococcus* strains whereas the homologous strain and usually a few other *Micrococcus* strains were still positive Therefore in addition to the shared antibodies micrococcal immune sera also have antibodies specific to micrococci Table 4 in which the results of absorptions of *M luteus* 1335 immune serum have been compiled illustrates this It can be deduced that serum 1335 has at least two antibodies common to micrococci and staphylococci and at least two additional antibodies apparently specific for micrococci

TABLE 3

Agglutination in M luteus 1335 Immune Serum

	No of strains tested	No of strains agglutinating in Serum dilution							
		1	10	20	40	80	160	320	640
Micrococcus	18	13	11	8	4	2	2	1	1
Staph aureus	19	13	5	4	1	0	0	0	0
Staph epiderm	8	4	2	2	1	0	0	0	0

Reciprocal value
§ Homologous strain

TABLE 4

Absorptions of M luteus 1335 Immune Serum

	No of strains tested	No of strains agglutinating in *M luteus* 1335 serum				
		Before absorption	After successive absorptions with *Staph aur* W46	*Staph aur* 830	*M conglom* 740	*M luteus* 210
Micrococcus	18	13	7	4	2	0
Staph aureus	19	13	2	0		
Staph epidermidis	8	4	1	0		

Strong agglutination with *M luteus* 1335 and 210 weak with *M conglom* 740 and 84
Agglutination with *M luteus* 1335 and 210

The agglutinating and absorbing abilities of *Staph aureus* Wood 46 the strain used for the first absorption in these experiments were not destroyed by autoclaving or treatment with trypsin Thus the most widely distributed of the agglutinogens common to micrococci and staphylococci do not seem to be of protein nature

Agglutination in Staph aureus factor sera The agglutinogens common to micrococci and staphylococci have been interpreted above as group antigens To make sure that the cross reactions were not due to the presence of specific *Staph aureus* agglutinogens in micrococci the

Micrococcus strains were agglutinated in our set of 13 *Staph aureus* factor sera The majority of the *Micrococcus* strains agglutinated in one or two factor sera but the reactions were weak and scattered They were interpreted as resulting from preimmune antibodies against micrococci These antibodies had not been removed by the *Staph aureus* strains used for absorption because they lacked the corresponding antigens (see above)

Specific Micrococcus agglutinogens The presence of specific micrococcal agglutinogens and the antigenic relationship between the *Micrococcus* strains were evaluated by titrations of the agglutinins of the *Micrococcus* immune sera and by successive absorptions After each absorption a decreasing number of Micrococcus strains was agglutinated and when a serum had been absorbed with 3 or 4 strains an antibody remained which in most instances agglutinated the homologous strain exclusively

TABLE 5
Micrococcus Strains with Similar but not Identical Patterns of Agglutinogens

Group 1	*M luteus*	810
		210
		1335
Group 2	*M roseus*	861
		691
	M luteus	144
Group 3	*M conglom*	740
		84
		547
	M varians	584
Group 4	*M varians*	1176
		719

Like *Staph aureus* the micrococci seem to have a number of specific agglutinogens The individual strains share some of these agglutinogens in different combinations and can be characterized by patterns of antigens No agglutinogens characteristic for *Micrococcus* as a genus or for any of the four species of this material could be demonstrated Some of the *Micrococcus* strains however were clearly related in their content of agglutinogens although none of them had exactly identical patterns Table 5 shows groups of serologically related strains There is a tendency towards a correlation between the species and the groups according to agglutination But of the 12 *M luteus* and *M roseus* strains only 6 are found in groups 1 and 2 whereas the others have different patterns of agglutinogens

Precipitation

Ring test precipitation was performed with extracts of the *Micrococcus* strains against some *Staph aureus*, *Staph epidermidis* and *Micrococcus* immune sera. This screening gave quite distributed precipitations but not such frequent reactions as agglutination. Cross precipitations occurred between *Staph aureus*, *Staph epidermidis* and micrococci. More specific reactions were not to be expected with these crude antigens.

Agar gel precipitation. All but two *Micrococcus* strains produced one or more precipitation lines against the homologous immune serum. Of these all except two strains also produced lines against one or more heterologous *Micrococcus* immune sera. The following precipitation lines were identified by means of known reference systems: four strains produced a polysaccharide A line (beta glucosaminyl ribitol teichoic acid) and 3 strains an I 3 line (alpha glucosaminyl glycerol teichoic acid) (Table 6). The precipitation lines did not show any correlation with the species or with the results of agglutination.

TABLE 6
Agar Gel Precipitation Lines Identified

Polysaccharide A line	*M conglomeratus*	84
	M varians	719
		1176
	Staph epiderm	[illegible]
I 3 teichoic acid line	*M luteus*	144
	M roseus	142
	M varians	1176

The *Staph aureus* precipitinogens polysaccharide *263* (alpha glucosaminyl ribitol teichoic acid), protein A and antigen D were not demonstrated in any of the *Micrococcus* strains. Absorptions were not carried out.

Haemagglutination

Extracts of all the *Micrococcus* strains and some *Staph epidermidis* strains were tested for their ability to sensitize normal and tanned sheep erythrocytes. When he treated erythrocytes were examined in dilutions of *Staph aureus* Cowan I immune serum, all *M luteus*, *M roseus* and *M conglomeratus* strains gave a negative or doubtful agglutination, whereas all three *M varians* strains, the two strains (*M conglomeratus* 107, *M varians* 529), which were shown to belong to *Staph epidermidis*, and all the other *Staph epidermidis* strains examined gave a strong haemagglutination. This indicates that *M luteus*, *M roseus* and *M conglomeratus* lack the heterogenetic antigen sensitizing normal erythrocytes and the staphylococcal antigen sensitizing tanned cells, whereas *M varians* has them.

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IN VITRO STUDIES OF SOME IMMUNOLOGICAL PHENOMENA IN EXPERIMENTAL RABBIT TOXOPLASMOSIS

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Received 23 i 67

It is well known that in many hosts toxoplasmosis is accompanied by the development of both circulating antibodies and delayed sensitivity This relationship is the case in man and in a number of laboratory animals *e g* the rabbit Animals which have passed an infection caused by a strain of low virulence are protected against an otherwise fatal disease if exposed to a virulent strain On the other hand rabbits sero positive after immunization with killed Toxoplasma organisms suc cumbed after challenge with the virulent RH strain even when anti body titres were high These animals were toxoplasmin negative while rabbits with high post infection antibody titres were positive (16) However the relationship beween the demonstrated immune reactions and protection to challenge is not clear

Phytohaemagglutinin was first shown by *Nowell* (23) to induce blastogenesis in peripheral blood leukocytes *in vitro* This observation later was confirmed and evidence presented that the blood cells af fected by phytohaemagglutinin were small lymphocytes (23) Other agents such as staphylococcal filtrate and streptolysin S were reported to cause nonspecific blastogenesis (12 20 27 28) Specific antigenic stimulation of lymphocytes from previously sensitized donors also has been reported for a number of antigenic substances (23) the most ef fective of which seems to be purified tuberculin protein derivative (PPD) Specific stimulation also can be induced with rabbit anti lymphocyte serum (8 10 27 28) and with homologous lymphocytes from an unrelated donor (2 26)

Little is known about the mechani m of blastogenesis after stimula tion with these ag nt or of the fu ion of the transformed cells So far no convincing da t have been p nt d to prove any fundamental difference in bl stog n sis after nons ific and specific stimulation No histologic or metabolic diffe nce between blastoid cells formed after stimulation w th the different agents have been reported Although some rese r hers have attributed immunological properti s

to the transformed cells (1 6 24) no other function than mitosis has been adequately proven (24) —However it has been claimed that the *in vitro* formed blastoid cells closely resemble the large pyroninophilic cells found *in vivo* in homografts and graft versus host reactions and also the blast cells found in lymphatic tissues after antigenic stimulation (24)

Preliminary investigations showed that both toxoplasmin and living Toxoplasma can stimulate transformation of sensitized rabbit lymphocytes Therefore a comparison of the ratio of blastoid cells after stimulation of peripheral blood lymph node and spleen lymphocytes might give an idea of the existence and distribution of sensitized immunocompetent cells

The capacity of the Toxoplasma organisms to invade cells and multiply *in the various cultures used was studied An attempt* was made to inhibit the infection of lymphoid cell cultures with high titre antisera from rabbits immunized with live or killed Toxoplasma organisms

MATERIAL

Rabbits Twenty four young healthy toxoplasmin and sero negative (dye test complement fixation test) rabbits weighing around 2 5 kg were used The rabbits were kept as described earlier (14)

Toxoplasma strains Sabin s strain RH was used throughout for the production of antigen for the dye and complement fixation test and for toxoplasmin The same strain was used as a stimulant and infective agent for lymphocyte cultures. A non fatal infection of rabbits was obtained with the strain toxo 6°6 6°" The strain was originally isolated from a human case and obtained through the courtesy of Dr J Chr Siim of the State Serum Institute of Copenhagen

Toxoplasmin For stimulation of lymphocytes a purified preparation of strain RH was used Toxoplasmin prepared as described previously (16) was passed through a Sephadex G 200 column in 0 1 M Tris-HCl 0 5 M NaCl ° per cent butanol buffer pH 8 0 Toxoplasma specific antigenic activity was demonstrated only in the high molecular peak and the antigen could thus be separated from the main part of extraneous protein

The crude preparation together with the control antigen described earlier (16) were used for skin tests

Non Specific stimulant Phytohaemagglutinin (PHA) Wellcome batch K639 was used

METHODS

Immunization of rabbits Twelve rabbits were inoculated in the foot pad with 0 5 ml of a suspension of brains from 5 mice infected with the toxo 6 ° 6 " strain *After 3 4 weeks sera were positive to high titres in both dye* test and complement fixation test however the toxoplasmin reaction was not positive until 5 8 weeks after inoculation Five rabbits were immunized with killed Toxoplasma organisms as described in a previous paper (15)

Serological examination Dye test (DT) and complement fixation test (CFT) were performed as described earlier (13)

Lymphocyte Suspensions

Peripheral blood Blood was obtained by cardiac puncture and defibrinated with glass beads Gelatin fractionation of the cell population was carried out according to the procedure described by *Coulson* & *Chalmers* (3) The cells were washed once in Parker s medium 199 containing 300 IU penicillin and 100 gamma strep

tomycin per ml and resuspended The concentration of lymphocytes was determined with a haemocytometer and their viability tested with trypan blue The suspensions were adjusted to 2×10^6 lymphocytes per ml Less than 1 per cent of the lymphocytes were non viable

These suspensions always contained erythrocytes in about the same concentration as leukocytes Of the leukocytes 90–95 per cent were lymphocytes 1–2 per cent were monocytes and the remaining cells were granulocytes

Lymph node and spleen The lymph node cell suspension was obtained from the two popliteal lymph nodes Lymph node or spleen was cut into small pieces and passed through a Borel sieve into 5–10 ml Parker s medium After centrifugation washing once and viability test the concentration was adjusted to 2×10^6 viable cells per ml

The cell suspensions from lymph nodes contained 70–80 per cent lymphocytes Most of the remaining cells were macrophages About 10–20 per cent of the cells mostly macrophages and large lymphocytes were non viable

The spleen cell suspension contained macrophages in the same proportion as the lymph node cell suspensions About 5–15 per cent of the cells were non viable mostly macrophages and large lymphocytes

Cell cultures One ml of cell suspension was added to a 10×80 mm tube containing 0 3 ml fetal calf serum To this mixture was added 1 ml Parker s medium containing either PHA toxoplasmin or living Toxoplasma

The cultures were observed for 96 hours Incubated at 37 the number of live lymphocytes decreased with 10 per cent or less within this time

Cell preparations for microscopical examination The apparatus described by *Dore & Balfour* (5) was used Of the cell suspension 0 1 ml was taken from the bottom of a tube The cells were sedimented and flattened on a small area of a glass slide by centrifugal force Simultaneously the fluid of the suspension was drained by a filter paper surrounding this area This method gave good preparations with easily observed details in the separate cells The cell preparations were fixed in methanol and stained by the May Grunwald-Giemsa methods Five hundred lymphocytes were counted in each preparation

Toxoplasma suspensions Infected mouse peritoneal exudate contained free Toxoplasma organisms and a considerable number of macrophages many of which contained multiplying parasites A pure suspension of free parasites was obtained by the following procedure One–2 ml freshly harvested mouse peritoneal exudate was diluted with 6–8 ml Parker s medium and centrifuged for 15 minutes at 2000 G The supernatant was discarded and the cells resuspended in 6–8 ml Parker s medium The suspension was filtered through a millipore filter pore size 8 μ with a positive air pressure of 1 5 kg/cm No cells passed the filter howeve by turning the filter upside down the Toxoplasma organisms could be flushed out of the filter leaving the macrophages firmly attached By this technique a pure Toxoplasma suspension containing about 20 per cent of the o ginal number of organisms was obtained Viabil ty tests showed that 7 10 per cent of the toxoplasms were dead after the procedure compared to 3–5 per cent in the untreated exudate

EXPERIMENTAL AND RESULTS

Lymphocyte cultures were investigated from three groups of rabbits namely

1) Toxoplasmin and ero negative (5 animals)
2) Toxoplasmin negative but sero po itive in high titre (DT ≧ 1/1250 CFT ≧ 1/120) after immunization with killed Toxoplasma organisms (5 animal)
3) Toxoplasmin and ro positive (DT ≧ 1/1250 CFT ≧ 1/120) after infection with the toxo 626 62 strain (10 animals)

Five rabbit were inoculated 6 months before the experiment and five only 7–9 weeks prior to the investigation when the toxopl smin

reaction had become recently positive There was no principal difference in the reactions recorded in these two subgroups Therefore they were pooled for the transformation experiments whereas they are accounted for separately in the section on susceptibility to Toxoplasma infection for reasons that will be given later

Transformation ratio The following stimulants were added to the cultures

1) PHA in a final dilution of 1/100

2) The purified toxoplasmin preparation in a final dilution of 1/100 (16 experiments) Preliminary investigations had shown that this dilution gave optimal lymphocyte stimulating activity —The crude preparation in a final dilution of 1/10 (4 experiments) When used in these dilutions parallel tests showed almost identical transformation ratios for the two preparations

3) Toxoplasma organisms suspended in Parker's medium in four concentrations corresponding to doses of 10 10^3 10^4 and 10 parasites per 10^6 lymphocytes It was found that a dose of 10^3 was most suitable for studies of transformation although some parasite multiplication and release of antigen occurred Higher parasite concentrations often caused vigorous infection of the cultures leading to cell degeneration after 2 or 3 days and the lower concentration often seemed to have too weak an antigenic effect

In each set a control tube was included containing only lymphocytes serum and Parker's medium

On account of the limited number of cells that were obtained from each animal only two sets of cultures could be prepared Thus each tube had to serve for two consecutive samples (on days 1 and 2 or 3 and 4 respectively)

Cells were classified as transformed when showing marked enlargement diameter $\geqq 12$ pronounced basophilia of the cytoplasm and a light slightly acidophilic area close to the nucleus The nuclear chromatin also had to be dispersed and the nucleoli distinct These cells are morphologically similar to the haemocytoblast as described by *Fagraeus* (7) Cells showing doubtful transformation were not included

Besides the transformation of lymphoid cells typical changes were seen in macrophages in cultures with multiplying Toxoplasma Beginning 24 or 48 hours after inoculation the macrophages showed increased marginal basophilia of the cytoplasm and mitosis in $\leqq 3$ per cent of the cells Such cells resembled the blastoid cells but were easily distinguishable particularly because of the typically shaped nucleus with coarse chromatin

Transformations were seen after 24 hours and their incidence increased rapidly thereafter This change occurred both after stimulation with PHA and after antigenic stimulation Generally mitoses were not observed before 48 hours They were seen almost invariably in cul

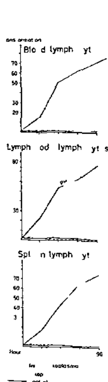


Fig 1

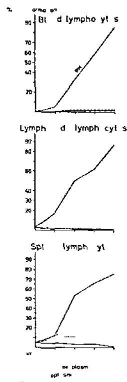


Fig 2

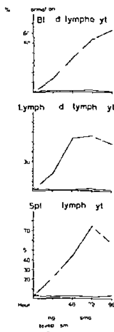


Fig 3

Fig 1 Transformation ratio in lymph cyte cultures from toxopla min and sero negative rabbit Mean percentage of blast forms from 4 readings during the observation period Measurements on day 1 performed immediately before the addition of stimulant to the cultu e

Fig 2 Transformation ratio in lymphocyte cultures from toxoplasmin negative but sero positive rabbits Mean percent ge of blast forms from 4 readings during the observation period Measurements on day 1 performed immediately be fore the addition of stimulant to the culture

F g 3 Transformation ratio in lymphocyte cultures from toxoplasmin and sero po itive rabbits Mean percentage of blast forms from 4 r adings during the observation period Measur m nts on day 1 performed immed atel b fore the addition of timulant to the culture

tures showing transformations but in small proportions corresponding to 1–5 per cent of transfo med cells

In Figs 1–3 the transformation rat o is indicated for peripheral blood lymph node and pleen lymphocytes for each group of rabbits As could be expected the transformat n ratio after PHA stimulation was very high

The statistical signif cance of th increase in antigenically stimu lated lymphocytes is indicated in Table 1 As can be seen a significant increase was d monstrated in lymphocytes from all three organ ori gins from both toxopl a min and sero-positive rabbits In rabbits toxo plasmin neg tive but ser positive the transformation ratio was signi

TABLE 1

Statistical Significance of Blastogenesis after Stimulation with Toxoplasmin and with Living Toxoplasma. The Average Percentage of Blast Forms on Day 1 (Immediately before Inoculation) and Day 4 are Compared Using Student's t test.—NS = not Significant

Rabbit group	Stimulant	Blood		P	Lymph node		P	Spleen		P
		Day 1	Day 4		Day 1	Day 4		Day 1	Day 4	
Seronegative	Toxoplasmin	1.0 ± 0.77	1.8 ± 0.58	NS	1.7 ± 0.60	1.0 ± 0.22	–	1.5 ± 0.50	2.5 ± 0.93	NS
	Living toxoplasms	1.0 ± 0.27	0.6 ± 0.13	–	1.7 ± 0.68	3.0 ± 1.03	NS	1.5 ± 0.50	5.1 ± 0.72	0.01
Toxoplasmin negative										
Seropositive	Toxoplasmin	0.1 ± 0.10	0.9 ± 0.30	0.05	2.3 ± 0.66	2.8 ± 0.80	NS	2.4 ± 1.01	20.2 ± 2.81	0.001
Toxoplasmin negative	Living toxoplasms	0.1 ± 0.10	12.4 ± 0.18	0.001	2.3 ± 0.66	2.5 ± 0.47	NS	2.4 ± 1.03	29.8 ± 5.98	0.01
Seropositive	Toxoplasmin	0.6 ± 0.13	10.7 ± 0.94	0.001	1.4 ± 0.32	14.4 ± 0.96	0.001	3.6 ± 0.73	26.6 ± 3.62	0.001
Toxoplasmin positive	Living toxoplasms	0.5 ± 0.15	14.4 ± 1.20	0.001	1.9 ± 0.64	17.3 ± 4.45	0.01	3.3 ± 0.73	35.6 ± 4.81	0.001

ficanly increased in spleen but not in lymph node lymphocytes In peripheral blood lymphocytes blastoid cells were present in very low proportion before stimulation During the observation period the ratio of such cells increased to values of only around 1 per cent Although the increase was statistically significant for both stimulants the number of transformed cells was very low and can hardly reflect any specific biological reaction In lymphocytes from toxoplasmin and sero negative rabbits a slightly significant increase in blastoid cells was demonstrated for spleen lymphocytes after stimulation with living Toxoplasma The data do not permit any explanation for this finding

From Figs 1–3 and Table 1 can be seen that in all samples the percentage of blastogenesis was somewhat higher when living parasites were used as a stimulant instead of toxoplasmin

Susceptibility of the Various Lymphocyte Cultures to Toxoplasma Infection

It was considered advisable to compare in the same experiment the lymphocyte cultures from rabbits belonging to the different experimental groups For practical reasons however it was not possible to examine cultures from more than two rabbits simultaneously The investigation was divided therefore into two main experiments each including lymphocyte cultures from five pairs of rabbits grouped as follows

Experiment I

1) Toxoplasmin and sero negative rabbits (group =)
2) Rabbits toxoplasmin and sero-positive after infection 6 months earlier (group + +)

Experiment II

1) Rabbits toxoplasmin negative but sero-positive after immunization with killed parasites (group ±)
2) Rabbits toxoplasmin and sero positive after infection 7–9 weeks earlier (group + +)

Cultures were classified as infected when multiplying Toxoplasma organisms were found inside one or se eral cells in microscopic fields of view containing a total of 500 lymphoc t s on one of the first three days and the same or corresponding culture proved to be infected on the following day also

Blood Lymphocyte Cul res

Table 2 shows the degree of infection 24 hours after inoculation The values are present d in a ratio of the number of infected cells per 1000 lymphocytes over th number of infected cultures After 24 hours

neither ruptured host cells nor extracellular Toxoplasma organisms were seen and the ratio of infected to total cells will approximate therefore the proportion of Toxoplasma organisms in the inoculum which had penetrated into cells. Table 2 shows that infected cells were found only in cultures from toxoplasmin negative rabbits and that such cells were few in numbers.

TABLE 2

Mean Number of Infected Cells per 1000 Lymphocytes in Infected Cultures over Number of Infected Cultures at 24 Hours after Inoculation. Groups of Rabbits Indicated as Follows: = Toxoplasmin—and Sero negative ++ Toxoplasmin—and Sero Positive after Infection 6 Months Earlier —+ Toxoplasmin Negative but Sero Positive after Immunization with Killed Parasites # Toxoplasmin—and Sero Positive after Infection 7–9 Weeks Earlier

Exp	Rabbit group	Blood No toxopl/10⁶ lymphoc		Lymph node No toxopl/10⁶ lymphoc		Spleen No toxopl/10⁶ lymphoc	
		10	10^4	10	10^4	10	10^4
I	=	5/2	2/1	12/4	5/2	6/3	6/2
	++	0/0	0/0	7/4	0/0	5/4	3/2
II	—+	9/1	0 0	35/5	14/5	39/4	10/3
	#	0/0	0/0	3[illegible]/5	11/5	20/3	5/2

TABLE 3

Lowest Infective Dose Giving Demonstrable Infection at 96 Hours after Inoculation. Groups of Rabbits Indicated as Follows: = Toxoplasmin—and Sero Positive after Infection 6 Months Earlier —+ Toxoplasmin Negative but Sero Positive after Immunization with Killed Parasites # Toxoplasmin—and Sero Positive after Infection 7–9 Weeks Earlier

Exp	Rabbit group	Blood Not inf	Blood No toxopl/10⁶ lymphoc		Lymph node Not inf	Lymph node No toxopl/10⁶ lymphoc		Spleen Not inf	Spleen No toxopl/10⁶ lymphoc		
			10	10^4		10^3	10		10	10^3	10
I	=	1	1	3	0	3	2	0	1	1	2
	++	4	1	0	0	1	3	0	1	3	0
II	—+	1	3	1	0	1	4	0	0	1	4
	#	3	0	0	0	1	4	0	0	[illegible]	3

Table 3 demonsrates the lowest Toxoplasma concentration that gave demonstrable infection during the observation period. In the two groups of toxoplasmin positive rabbits cultures from only one of 10 animals were found infected whereas in toxoplasmin negative rabbits cultures from 8 of 10 animals were infected. Infection could be observed only with a large inoculum of Toxoplasma however the diffe

rence between toxoplasmin positive and negative rabbits was significant ($P < 0\ 001$)

The parasite multiplication and spread within the cultures was limited Daily countings covering the entire observation period revealed only a low number of infected cells With an infective dose of 10 parasites per 10^6 lymphocytes a total of 49 Toxoplasma infected cells (22 macrophages 10 blastoid cells 17 small lymphocytes) were found in cultures from toxoplasmin negative rabbits whereas the corresponding figures for toxopasmin positive rabbits was only 5 (2 macrophages 3 small lymphocytes) In no preparation was Toxoplasma found within a pseudo eosinophilic leukocyte

Lymph Node and Spleen Cultures

The data in Tables 2–3 indicate that the infection rate was far greater in cultures from these organs compared to peripheral blood Table 2 shows that when the infective dose was 10 parasites per 10^6 lymphocytes approximately 5–39 per cent of inoculated parasites had penetrated into cells after 24 hours A higher percentage was found with an infective dose of 10^4 organisms The fact that in a number of cultures the calculated ratio exceeded 100 per cent might be accounted for by random variation or it might indicate that a second cycle of Toxoplasma growth had occurred Table 3 shows that infection of cells also followed upon inoculation of small numbers of Toxoplasma

No significant difference was demonstrated between cultures from immune and susceptible animals The ratio of infected cells was somewhat higher throughout in experiment II than in I but this was probably due to technical factors

In Figs 4–6 an attempt was made to analyse further the difference in susceptibility to Toxoplasma infection between blood lymphocyte cultures and cultures from lymph nodes and spleen —Fig 4 shows the distribution of different cells in the cultures immediately before inoculation with Toxoplasma The blood lymphocyte cultures consisted almost entirely of small lymphocytes Large lymphoid cells and macrophages were less than 1 per cent of the population Pseudo eosinophilic leukocytes were found in a mean ratio of 3 per 100 lymphocytes Cultures from lymph nodes and spleen contained macrophages in ratios of about 20 per 100 lymphocytes as well as small lymphocytes and a small number of large lymphoid cells

In Fig 5 is shown the ratio of infected macrophages blastoid cells and small lymphocytes in lymph node cultures 24 hours after infection with 10 parasites per 10^6 lymphocytes The largest ratio of infected cells 11–29 per cent was found in the macrophage group 2–10 per cent of the blastoid cells were infected whereas infection of small lymphocytes was rare 0 1–1 per cent The results are representative for spleen cultures and for lymph node and spleen cultures infected

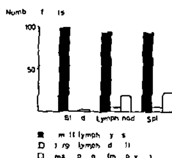


Fig 4

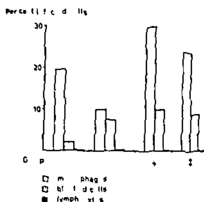


Fig 5

Fig 4 Proportion of small lymphocytes large lymphoid cells and macrophages in blood lymph node and spleen cultures before addition of Toxoplasma Data presented correspond to an average of all cultures used in the investigation

Fig 5 Lymph node cultures 24 hours after inoculation with 10^5 Toxoplasma organisms per 10^6 lymphocytes Percentage of Toxoplasma infected cells in macrophages blastoid cells and small lymphocytes in the two experiments and four rabbit groups

with 10^4 parasites per 10^6 lymphocytes It therefore seems evident that the macrophages were of fundamental importance for the early multiplication of Toxoplasma in the cultures However it is also clear that a fairly large proportion of blastoid cells were infected (Fig 6) As the ratio of blastoid cells in several cultures especially from the spleen increased considerably during the observation period it was presumed that the late propagation to a large extent occurred in such cells Fig 7 presents the ratios of infected macrophages and blastoid cells from all types of cultures 48 hours after inoculation with 10^5 and 10^4 toxoplasms per 10^6 lymphocytes At this time the number of infected cells had increased considerably indicating secondary infection by toxoplasms released from initially infected cells The figure suggests that the increase was larger in blastoid cells than in macrophages however the difference was not statistically significant The percentage of infected small lymphocytes at this time was still $\leqq 1$ per cent 48 hours after inoculation the number of intracellular parasites was very large In many cases around 100 parasites were seen within one macrophage and 60 70 toxoplasms in one blastoid cell In both types of cells the parasites were often located in several clones No difference in the number of intracellular parasites was observed between cultures from immune and susceptible rabbits More than two parasites per cell were seldom seen within small lymphocytes

In order to investigate if the demonstrated susceptibility of blastoid cells to Toxoplasma infection applies to blastoid cells in general or only to antigenically stimulated transformed lymphocytes the ratio of infected cells in blood lymphocyte cultures treated and untreated with PHA 2 days earlier were investigated Twelve cultures from the same



Fig 6

Transformed lymph node lymphocytes containing multiplying Toxoplasma organisms

sero and toxoplasmin negative rabbit were inoculated 6 of which were unstimulated and 6 stimulated with PHA At the time of inoculation the ratio of blastoid cells was 0 2 per cent in the former and 43 per cent in the latter group Twenty four hours after inoculation with 10 Toxoplasma organisms per 10^6 lymphocytes the mean number of infected cells per 1000 lymphocytes was $11 \pm 2\ 9$ in the unstimulated and $121 \pm 6\ 0$ in the stimulated group 83 per cent of the infected cells in the stimulated cultures were blastoid forms The difference between the two groups is statistically significant $P < 0\ 001$ Pseudo eosinophilic leukocytes were found in a frequence of 3 4 per cent in the cultures 9 per cent of these cells were found infected during the observation period After 24 hours the granulocytes frequently contained multiplying Toxoplasma however the parasites stained poorly and were

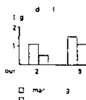


Fig 7

Percentage of infected macrophages and blastoid cells 48 hours after inoculation The values are calculated from the cumulative data from all lymph node and spleen cultures inoculated with either 10^4 or 10^5 Toxoplasma organisms per 10^6 lymphocytes

not clearly defined in the surrounding cytoplasm. From 48 hours after inoculation they were detected only with difficulty within such cells. Most probably they were being destroyed within these cells.

Inhibitory Effect of DT and CFT Positive Serum on Penetration and Multiplication of Toxoplasma

Since Toxoplasma multiplication was poor in blood lymphocyte cultures, only lymph node and spleen cultures were investigated. Lymphocytes from two rabbits toxoplasmin and sero positive (group ++) and two toxoplasmin and sero negative (group =) were tested.

Two DT and CFT positive sera were used. One from a rabbit that had passed an infection 3 months earlier (DT 1/6250, CFT 1/1920). The second serum was pooled from two rabbits immunized with killed parasites 3 months earlier (DT 1/1250, CFT 1/240). They were stored at −20 C and used within 4 weeks after bleeding.

The cultures were set up as described earlier. Instead of fetal calf serum 0.1 0.3 ml positive rabbit serum was added to the lymphocyte suspensions. The rabbit immune serum was used: 1) native 0.3 ml (n), 2) inactivated at 56 C for 30 minutes 0.3 ml (i), 3) 0.1 ml inactivated rabbit serum and 0.2 ml human accessory serum (a). Fetal calf serum was used as a control.

Toxoplasma suspensions were added in two concentrations 10^6 and 10^4 per 10^6 lymphocytes. Owing to the large number of tubes, lymphocyte cultures from only one animal could be examined in each experiment.

All control cultures were degenerated after 96 hours. The data in Table 4 demonstrate that both sera used had a protective effect. In a few cultures, however, where inactivated serum with or without accessory factor was added, this protection was overcome by the invading parasites, resulting in widespread infection and cell degeneration. Such infection occurred in one of 48 cultures in group ++ and in 6 of 48 cultures in group =. In a few more cultures, mainly in group =, a small number of infected cells were found, indicating an incomplete protection.

DISCUSSION

Small lymphocytes from toxoplasmin and/or sero positive rabbits were transformed into blastoid cells when stimulated with either toxoplasmin or living Toxoplasma organisms, whereas lymphocytes from toxoplasmin and sero negative rabbits did not show transformations to any significant degree (with the exception of spleen cells stimulated with living Toxoplasma). This result indicates that the transformations were the result of a specific stimulation of previously sensitized cells.

It was demonstrated that the percentage of blastoid cells in the population was larger after stimulation with living Toxoplasma than when toxoplasmin was used. The reason might well be that a greater number of antigenic substances was released in cultures containing living parasites.

TABLE 4

Inhibitory Effect of DT and CFT Positive Sera on Infection of Lymph Node and Spleen Cultures. The Data were Obtained from Observations 96 Hours after Inoculation. Serum P = Immune Serum Postinfectious. I = Immune Serum after Immunization with Killed Parasites. C = Control Fetal Calf Serum. n = Native. i = Inactivated. a = Inactivated + Human Accessory Factor. Number of Infected Cells per 1000 Lymphocytes/Number of Infected Cultures. Deg = Cultures Degenerated by Vigorous Toxoplasma Infection

Lymph Node Lymphocytes

	Group ++			Group =	
Serum	No. toxoplasma/10^6 lymphocytes		Serum	No. toxoplasma/10^6 lymphocytes	
	10^5	10^4		10^5	10^4
C	deg./2	deg./[illegible]	C	deg./2	deg./2
Pn	0	0	Pn	1/1	0
Pi	1/1	0	Pi	1/1 deg./1	deg./1
Pa	0	0	Pa	1/1 deg./1	deg./1
In	0	0	In	1/1	2/1
Ii	0	0	Ii	8/[illegible]	2/2
Ia	deg./1	0	Ia	6/[illegible]	0

Spleen Lymphocytes

	Group ++			Group =	
Serum	No. toxoplasma/10^6 lymphocytes		Serum	No. toxoplasma/10^6 lymphocytes	
	10^5	10^4		10^5	10^4
C	deg./2	deg./2	C	deg./[illegible]	deg./2
Pn	0	0	Pn	0	0
Pi	1/1	0	Pi	1/1 deg./1	deg./1
Pa	0	0	Pa	3 ›	2 1
In	0	0	In	0	0
Ii	0	0	Ii	[illegible]	[illegible]/1
Ia	4/1	0	Ia	31/[illegible]	0

Blastoid cells were found in considerably larger proportions in stimulated spleen cell cultures than in cultures from peripheral blood and lymph nodes. Possibly the spleen contained a higher percentage sensitized lymphocytes or the spleen lymphocytes were more reactive than lymphocytes from the other sources. It is well known (17) that intravenous injection of particulate antigens is followed by antibody production that takes place mainly in the spleen and to a smaller extent in lymph nodes. Under these conditions probably a larger number of lymphocytes were sensitized in spleen than in lymph nodes and peripheral blood. In the present experiment one group of rabbits was im-

munized intravenously with killed Toxoplasma while in the other group an early appearing parasitaemia (14) may have played a role similar to intravenous inoculation These conditions would favour the supposition that spleen cells were particularly sensitized On the other hand transformation after PHA stimulation was not observed to any greater extent in spleen than in peripheral blood and lymph node lymphocytes This observation does not support the idea that the spleen cells were more reactive than lymphocytes from blood and lymph node

In sensitized rabbits up to 35 per cent of spleen lymphocytes were found transformed after 96 hours It does not seem very likely that this reflects the number of lymphocytes originally sensitized The finding might to some extent be due to selective multiplication of sensitized cells However this phenomenon cannot entirely explain the large percentage of transformed lymphocytes The mechanism of blastogenesis is probably complex and it might involve stimulation of non sensitized cells as has been suggested by other investigators (11 27) No conclusions about this mechanism can be drawn from the present study

Blastogenesis was more pronounced in lymphocyte cultures from rabbits which became toxoplasmin and sero positive after infection than in cultures from rabbits sero positive only after immunization with killed parasites In the latter group a significant increase in blastoid cells was shown only in spleen lymphocytes The difference might be merely of a quantitative nature corresponding to a more intensive antigenic stimulation in the former group However it is known that the percentage of lymphocytes responsible for cell bound immunity is larger in peripheral blood and lymph nodes than in the spleen (22) Blastogenesis in peripheral blood and lymph node lymphocytes from toxoplasmin positive rabbits therefore might indicate the presence of cell bound immunity

A problem of considerable interest is the following Do small lymphocytes sensitized against Toxoplasma perform an immunological function of significance for immune protection? The role of small lymphocytes in the immune response to homograft and graft against host reaction has been established and reviewed by Gowans (9) However only a few studies have been reported so far indicating that small lymphocytes produce protective immunity against infective agents *Kempe* (18) reported a case of progressive vaccinia in a child with normal gamma globulin Massive doses of immune gamma globulin had no effect of the lesions but their progress was halted dramatically by injection of leukocytes from recently vaccinated donors in the neighboring skin *Larsh et al* (19) showed that peritoneal exudate cells obtained from a donor mouse infected with Trichinella spiralis caused the accelerated removal of adult worms when injected into a recipient Recently *Dineen & Wagland* (4) showed that cells obtained from mesenteric lymph nodes of inbred guinea pigs infected with Trichostrongylus colubriformis caused resistance when injected within eight

days before challenge inoculation The transfer of massive volumes of serum from resistant to susceptible animals was without effect

The present investigation does not give unequivocal evidence that sensitized small lymphocytes are involved in the mechanism of immune protection in toxoplasmosis *The observed difference in the proportion* of infected blood lymphocyte cultures obtained from toxoplasmin positive or negative animals was statistically significant but as the total number of infected cells was small the biological significance of this observation remains to be clarified

The data suggest that blastoid cells are more easily infected with Toxoplasma than untransformed lymphocytes During he entire observation period only 1 per cent or less of the small lymphocytes were found infected and 24 and 48 hours after inoculation 3 per cent and 14 per cent respectively of the blastoid cells were infected One possible reason for the greater susceptibility of blastoid cells might be an altered cell surface structure permitting easier penetration for the parasites The fact that blastoid cells are readily infected and support vigorous multiplication of the Toxoplasma organisms strongly suggests that in this stage of development the cells have no immune protective function

Of certain immunological interest are the macrophage cells present in the cultures It was demonstrated by *Vischer & Suter* (29) in experiments with cultures of peritoneal macrophages from laboratory rodents that Toxoplasma can be propagated successfully in such cultures and that the intracellular multiplication of the parasites was inhibited in macrophages from rats or guinea pigs actively immunized with living Toxoplasma The results of the present investigation are in agreement with the first observation In lymph node and spleen cultures a larger number of macrophages were found infected than would be expected from their distribution and their proportional mass This may be a result of the phagocytotic activity of the macrophages

The second observation by *Vischer & Suter* could not be confirmed No significant difference in the percentage of inferted macrophages or in the number of intracellular parasites was found between cultures from immune and susceptible animals Despite the fact that several authors have presented evidence to show that macrophages from immune animals have a specific ability to inactivate and destroy intracellular infective agents other researchers have not been able to demonstrate the same phenomenon (24) The importance of macrophages in the immune mechanism is still obscure

One cell type had an unequivocal destructive effect on Toxoplasma namely the granulocyt The rapid intracellular killing of the parasit by such cells has been demonstrated previously (15) and in this investigation This phenomenon which evidently is non specific in nat may be due to the action of proteolytic enzymes present in high concentration in the granule of these cells

Lycke et al. (21) have demonstrated that the penetration of Toxoplasma into cells *in vitro* could be inhibited by a mixture of immune serum and accessory factor as well as by the same mixture when the heat labile components had been destroyed. The results of the present investigation are consistent with these findings. Inhibition of Toxoplasma infection in lymph node and spleen cultures could be demonstrated both with native and inactivated DT and CFT positive serum.

The present investigation shows that DT and CFT positive serum from rabbits immunized with killed parasites had as effective an inhibitory effect as serum from a rabbit sero-positive after an active toxoplasmic infection. This finding suggests that the difference in susceptibility to challenge *in vivo* between rabbits immunized with killed Toxoplasma and rabbits with high post-infection antibody titres is not due to any fundamental difference in the antibody response against living and killed parasites.

A difference in the protective effect of DT and CFT positive serum between cultures from immune and susceptible animals was observed but the present limited investigation does not allow any further conclusions.

SUMMARY

The transformation to blastoid cells in lymphocyte cultures from peripheral blood, lymph nodes and spleen from rabbits with varying degrees of immunity were investigated after stimulation with toxoplasmin or living Toxoplasma organisms. The capacity of Toxoplasma to invade and multiply within these cells was studied also. Finally the inhibitory effect of DT and CFT positive sera was tested in cultures of lymph nodes and spleen.

A significant increase in blastogenesis during the observation period (96 hours) was demonstrated in all three types of lymphocyte cultures studied from rabbits toxoplasmin- and sero-positive after a passed infection. Lymphocyte cultures from rabbits toxoplasmin negative but sero-positive after immunization with killed parasites showed a significant increase in transformations only in spleen lymphocytes. In cultures from toxoplasmin- and sero-negative rabbits a significant increase in blastogenesis was not observed with the exception of a minimal although significant increase in spleen lymphocytes stimulated with living parasites. The percentage of transformations was larger after stimulation with living parasites than when toxoplasmin was used.

A difference in the proportion of infected blood lymphocyte cultures from susceptible and immune rabbits observed suggests but does not prove the existence of an immune protective property of lymphocytes.

After transformation the blastoid cells were infected more easily by Toxoplasma and multiplication was vigorous within such cells.

It was demonstrated that macrophages present to around 20 per cent

in lymph node and spleen cultures were easily infected with Toxoplasma and that multiplication was pronounced within them No difference either in the percentage of infected cells or in the number of intracellular parasites was observed between macrophages from immune and susceptible animals

Toxoplasma organisms were rapidly destroyed within granulocytes This activity of the host cell is probably nonspecific in nature

In vitro infection with Toxoplasma was inhibited with S serum with high post infection antibody titres and with a serum with high DT and CFT titres after immunization with killed parasites

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 2–3 1966

G M berger & J G Norlen A SEMINAR ON TUMOURS OF THE URINARY BLADDER

Fifty-one Swedish pathologists representing almost all of the departments of pathology of the country had diagnosed 15 slides Opinions on cytology were fairly uniform but conclusions with regard to degree of malignancy and infiltration differed widel The differences were more pronounced between than within the departments though an individual usually had a constant opinion in relation to the others

The conclusions were mostly based upon specified criteria but 5 different criteria systems had been used and this was probably in principle the cause of the differences in conclusions

Thirteen of the 15 slides were from patients who at follow up showed invasive cancer and the two others had extensive mucosal tumour growth and clinically malignant course Of the 765 submitted diagnoses 235 were benign 74 strong suspicion of malignancy and 456 definite cancer

Görel Östberg H Hedeland C B Hökfelt ON THE OCCURRENCE OF ADRENAL CORTICAL ADENOMA ESSENTIAL HYPERTENSION AND CHANGES IN GLUCOSE BALANCE IN AN AUTOPSY MATERIAL

Conn has suggested that about 20 per cent of the cases with so-called benign essential hypertension might have an aldosterone producing adrenal cortical adenoma Primary aldosteronism might also have a relation to lowered glucose tolerance

All the subjects aged 50 and above autopsied at the Institute of Pathology in Malmo during 6 months (730 cases) were studied for clinical diagnosis of hypertension and glycosuria (diabetes mellitus) and autopsy findings of adrenal cortical adenoma More adenomas were found in male hypertensives than in normotensives 18 9 and 8 4 per cent respectively (001 < p [illegible]) The [illegible] was identical in women with and without hypertension The [illegible] of adenomas was a little higher in diabetics than in non diabetics [illegible] ent respectively (01 < p < 0) but the difference was not [illegible]

According to this [illegible] the frequency of adrenal [illegible] in hypertension and diabetes was [illegible] than [illegible] frequencies previously published [illegible] frequency of adenomas however [illegible] rather high 10 1 per cent in the men and 6 per cent in the women

J Wajerman K A M berg A Kristina Stenhjem ACUTE IDIOPATHIC PANCREATITIS—A VASCULAR DISEASE?

I B Schnürer HISTOPATHOLOGICAL CHANGES IN TANDERIL® INDUCED PAROTITIS

In 1965 five patients were observed in whom fever enlarged salivary glands and a feeling of dryness in the eyes and the mouth developed after treatment with Tanderil (oxifenbutazon)

A woman of 66 who for 10 days had consumed in all 30 tablets Tanderil (3 grams) ran a fever and the parotid glands on both sides swelled Sialographic examination did not reveal anything abnormal A biopsy about 14 days after she had been taken ill showed periductal inflammatory infiltration composed predominantly of lymphocytes with occasional reticulocytes forming small germinal centres The acinic part of the gland showed only a moderately pronounced oedema

H Knutson Allan Lunderquist & Anders Lunderquist DIFFERENTIAL DIAGNOSIS BETWEEN ULCERATIVE COLITIS AND GRANULOMATOUS ENTEROCOLITIS (CROHN S DISEASE) PATHOLOGICAL AND ANGIOGRAPHICAL ASPECTS

In a small number of cases the superior and/or inferior mesenteric artery had been studied preoperatively by angiography In all the cases of *Crohn s* disease obliterative vascular changes in the small and medium sized intestinal vessels were observed but were found only occasionally in ulcerative colitis

The vascular changes were regarded as non specific but as they were always found in *Crohn s* disease they were thougt to be of diagnostic importance and an indication of a chronic disease The angiographic picture in advanced stages of *Crohn s* disease differed from that in ulcerative colitis in that the former exhibited irregularities in the distribution and the calibre of the arteries obliterations of the vessels and reduced venous return In addition to its value as a means by which to make a differential diagnosis possible between ulcerative colitis and *Crohn s* disease it permits an earlier and more precise localization of the extent of the disease than otherwise obtainable by the usual barium contrast method

C Hultquist C V O Bjork OBSERVATIONS IN BIOPSIES OF THE MYOCARDIUM IN CASES UNDERGOING OPEN HEART SURGERY

From cases with congenital heart disease or acquired valvular disease biopsies of the myocardium were taken with a hollow sharpened drill during open heart surgery before and after perfusion The accordance of the 88 double biopsies which were studied by light microscopy was found to be great concerning diffuse changes such as hypertrophy but only about 50 per cent in cases of focal lesions such as scar tissue As to interstitial oedema it was more often increased after than before perfusion which indicates that oedema in some cases might be an effect of the perfusion

Eighteen cases were studied with electron microscopy and enzyme histochemistry In 5 cases slight ultrastructural changes consisting of glycogen depletion and oedema of the sarcoplasma were observed In another 3 cases changes were more severe also including mitochondrial abnormalities such as swelling loss of cristae and matrices) margination of nuclear chromatin swelling and vacuolization of sarcoplastic reticulum and slight myofibrillar changes For dating these changes staining for phosphorylase succinodehydrogenase and cytochrome oxidase were of value In one case with severe changes it was thus made probable that they had started before the perfusion

The slight changes appeared in 3 cases of congenital heart abnormalities and 2 of acquired aortic valvular lesions whereas the severe changes were found in 3 cases of acquired aortic valvular lesions Among the 10 cases without ultrastructural changes 7 were congenital and 3 acquired heart lesions

J Pontén LYMPHOBLASTOID TRANSFORMATION IN CULTURES OF HUMAN MALIGNANT LYMPHOMAS

Lymphoid tissue from 6 cases of malignant lymphoma and 5 non malignant lymph nodes were grown in a modified grid monola er culture The control tissue after a preliminary phase of release of preformed lymphoid cells gave rise to regular monolayers of fibroblastoid morphology They could be subcultivated throughout about 10 passages before reaching a terminal degenerative stage The grids could be transferred ? 5 times before losing their capacity to produce fibroblastoid cells.

The six malignant tissue specimens ceased to produce lymphoid cells after the same length of time as the controls and also formed fibroblastoid monolayers The m nolayers deri ed from the first 2 grid transfer generations did not differ from the control monolayers Monolayers generated from later grid transfer generations displayed a pathological irregular growth pattern In 3 cases reticulo l elements appeared which differentiated into lymphoid cells 8–19 weeks after initiation of the cultures The reticulolymphoid cells could not be maintained in the ab ence of fibroblastoid cells but co cultivation with normal fibroblasts restored the capacity for survival of the reticulolymphoid cells Chromosome analysis of one transf rmed strain howed 47 chromosomes interpreted as a trisomy of no 11 or 12

The findings suggest a specific correlation between irregular growth and lympho blastoid transformation on one hand and malignant l mphoma on the other The cor relation cannot be considered definite however because of the small number of c ntrols and the difficulty in growing non malignant lymphoid tissue during a sufficiently long time It is not excluded that normal lymphnodes would show the same alterations if carried thr ugh as many gri l transfers as the malignant tissue

P Biberfeld C Holm & J Ericsson POLYSOME FORMATION AND MICROPINO CYTOSIS IN *IN VITRO* TRANSFORMED HUMAN LYMPHOCYTES

The light and electron microscopic appearance of hum n lymphocytes i olated from peripheral blood and cultured *in vitro* in the presence of phytohaemagglutinin (PHA) staphylococcal extract (STE) and tuberculin (PPD) was in estigated

Two hours after addition of PHA electron microscopy showed some small lympho cytes containing cytoplasmic ribosomes agg egated t t pi al poly(rib) ome The amount of polysomes and the number of cells ith these elements increased with time After 24 hours of PHA stimulation most cells c ntained numerou l ol om Counting of ribosomes sho ed a greater variation of rib s m l concent tion in transformed cells (?00–4 0/µ) than in unstimulated cells (3 0–45?) le l changes (enl rgement altered interrelationships of granular fib llar an l r phous material) were noted in some cells within 24 h urs afte PHA i mulat n

In transformed cells acid pho phatase activity—demonstrated b incub ti n in a Gomori type medium—was found in the Golgi area and in foot p end and was confined to single membrane limited bodie (l res and oc nal Golgi elements Untransforme l lymphocytes showed less or no n e a t it During mitosis centrifugal" redistribution of lysosome l ke bodies w s b e ed

Ferritin molecules introduced in the cultivation medium were ab o b d by mi ro

pinocytosis and were segregated in acid phosphatase positive bodies mainly multi vesicular bodies Micropinocytosis was greater in stimulated than in unstimulated cells

Lymphocytes from a Mantoux positive donor transformed by LHA STF and PPD in parallel experiments showed essentially similar ultrastructural features However the transformation time was longer and the rate of transformation lower with PPD than with STF and LHA

S Lindgren CONGENITAL PRIMARY ADRENAL HYPOPLASIA

At gross examination post mortem of a boy born at term who died from aspiration during delivery absence of the adrenals was observed Histological examination of serial sections of retroperitoneal tissue however revealed 2 very small adrenal The permanent cortex was very thin on the left side and almost absent on the right side The foetal cortex was predominant and consisted of irregularly arranged eosinophilic cells and numerous giant cells

The case was diagnosed as congenital primary hypoplasia of the cytomegalic type which should be distinguished from secondary hypoplasia in anencephalia *Eleven such cases were reported in the literature among them three pairs of brothers The* condition therefore appears to be a hereditary anomaly which soon gives symptoms of cortical insufficiency Two of the 11 patients aged 1 and 3½ years respectively are receiving substitution therapy and are alive

I Mikulowski SPERMATOCYTIC SEMINOMA

Up to now 32 cases of spermatocytic seminoma have been described Differences from usual seminomas include the high mean age of the patients (57 years) the tumours have been found only in the testis and always in a pure form absence of lymphocytic infiltration in the stroma and granulomatous reaction is not present

In a study of 94 testicular tumours of which 84 primarily had been classified as seminomas and 13 as sarcomas 7 (7 4 per cent) cases of spermatocytic seminomas were found In spite of tumour invasion into large blood vessels the prognosis seems to be relatively good Of the 7 cases 4 are living and well 2 of the cases have been observed for 5 and 4 years respectively after operation Two died from metastases after 6 5 and 4 years duration of disease respectively All the cases had been operated and had received X ray therapy postoperatively

In 22 cases of ovarian dysgerminoma no differentiation in the direction of spermatocytic seminoma was found

Germ cell tumours without sex differentiation should be called gonocytoma or germinoma The term seminoma (with the possible addition spermatocytic) should be reserved for tumours of cells of differentiated male type

Ingrid Hajerstrand *C B Ohlund* ASBESTOS AND MESOTHELIOMAS

In 1907 the first case of pulmonary fibrosis due to inhalation of asbestos fiber was reported During the last 30 years many reports have appeared about the high incidence of pulmonary tumours in asbestos workers and since 1960 the interest has been focused on the possible relation between asbestos exposure and malignant mesotheliomas of pleura and peritoneum

The results of the examination of 33 mesotheliomas did not support the idea of a relationship between asbestos exposure and mesothelioma This problem must still be considered unsettled

Th Berge *T Sall en* METASTATIC CARCINOMA IN THE CIRRHOTIC LIVER

Among 3 642 extrahepatic carcinomas portal cirrh sis was found c incidentally in 108 5 8 per cent of the carcinomas in the cirrhoti and 68 9 per cent in the non-cirrhotic group had metastasized among these 40 4 per cent and 51 6 per cent re pectively had metastases in the liver This difference which is not statisticall significant, could be explained b the fact that mrst f the tumours in the cirrhotic group were in an earl stage of disseminati n and had metastases only in a few organs Among the ca es with metastases t more than f ur organs all the tumours in the cirrhotic group ha l metastosized t the liver but the frequenc in the non cirrh tic group was b ut 80 per cent When attention was paid to the haemodyna mi p ssibilities of tumours in diffe ent l cations and the stage f dissemination the cirrhotic liver wa found to be just a g d a s il (pr bably better) for meta ta is as the normal liver

Fen Brehmer Anderss n & L Brunk TAPE STRIPPING METHOD FOR CYTO LOGICAL DIAGNOSIS OF MYCOSIS FUNGOIDES

A piece of rdinar transparent tape was applied t the affected skin area and then stripped off Thi was repeated using fresh tape each time until punctate oozing appeared A slide was held against the oozing kin f r about 20 seconds To btain material from varying depths of the lesi n stripping as continued and smears taken after every 10 tapings The material was fixed in 95 per cent al ohol and stained with May r s haematox lin and eosin The smears were alwa s taken from lesions which were covered b a macro c pically intact epidermis

The tape method was carried ut on 7 patients with clinically diagnosed m cosis fungoides and the re ults fr m 5 f the cases were presente l

The imprint smear c ntained numerous cell pre erved c ll permitting anal si of the configuration of the nuclei and the distribution of the chromatin In all case the smears showed distinctl at pical cells giving a definite diagn si of malignancy In two of these c ses repeate l l i sies had only given a pr on l li gno is of malignant di ea e

M Nasiell ABNORMAL COLUMNAR CELL FINDINGS IN BRONCHOGENIC CARCINOMA

According to *Papani l u* cili cyt phth ria (CCP) me ns degenerati n and destructi n f large numbers f b nchial column r cell *I pani l ou* f und an incr ased CCP incid nce in l ing cancer

In our sputum cytologic l tudies un h racteri ti de en r ti it g blet cell changes of the o umnar cells were fo nd as wel a th t c f hanges All these abnorm l c lumnar cell fin l ngs w call d ACI l gic l stud es sl t format on in the re pirat r muc s an l c t l urface cells was foun l in other e unalt r d c lumr s u r p bably c rrespond t the ACCF D fferent p tu c f l r t the cyt logical c lumn r cell alte ati ns w e f un c t Th lt of the process is a l w thelium without cilia wh n t n gainst exogeni

Th cytologic l l ist l g cal alte t ns sh a d t nn t r with meta l stic changes in th l onchial ep thelium as well a situ b n l carcinoma It quite possible t the low ep thelium without il whi n app e s after th

surface cell expulsion becomes the starting point of the metaplastic process which in turn shows a close association with bronchial carcinoma

S Dahlgren ASPIRATION BIOPSY OF INTRAPULMONARY HAMARTOMAS

P S Persson O Obrant C C Ahren CYTOLOGICAL AND HISTOLOGICAL EXAMINATION OF THE TESTIS IN AZOOSPERMIA

D Schiffer (Turin Italy) HISTOENZYMOLOGY OF NORMAL AND PATHOLOGICAL GLIA

B Lagerlöf CHROMOSOME PATTERN AND BIOLOGICAL PROPERTIES OF THE CELLS OF THE THYMUS DURING LEUKAEMOGENESIS

K Norrby F Knutson C P M Lundin CELLULAR DISSOCIATION IN ENZYMATICALLY PRODUCED TUMOUR CELL SUSPENSIONS

Cell suspensions produced enzymatically using trypsin in combination with DNase have high initial cellular viability and are generally considered fully dissociated In experiments on tumour kinetics a complete dissociation is desirable as hypotheses have been presented indicating aggregation or close contact between tumour cells as a prerequisite for malignant proliferation in vivo possibly with the exception of certain tumour cells in advanced progression However in enzymatic suspensions from an experimental mouse sarcoma two independent phenomena may appear resulting in clumping or aggregation of cells during the final resuspension One is the formation of a DNase sensitive gel in which cells are entrapped The other phenomenon is a spontaneous instant aggregation of cells in synthetic media This aggregation was interpreted as a type of primary aggregation described by Moscona

To achieve complete degradation of the extracellular DNase sensitive gel (probably DNP gel) in suspensions rich in cells several times the concentration of DNase recommended for enzymatic techniques was needed The amount of DNase required probably varies from tumour to tumour

The instant spontaneous aggregation in synthetic media was inhibited and already formed aggregates were dissociated by sera from various species and murine cell free ascitic fluid

F Lihl G Gustafson B Josefsson C K C Paul ULTRASTRUCTURAL LOCALIZATION OF HEAVY METALS IN EOSINOPHILIC GRANULOCYTES

Using a previously described modification of the sulphide silver method for ultrastructural studies (*Lihl & Falkmer 1967*) heavy metals were found in the specific granules from rat and cattle eosinophilic granulocytes All other organelles of both types of eosinophilic granulocytes gave a negative reaction The characteristic central crystalline structures of the eosinophilic granules also appeared to be devoid of heavy metals

As the modified sulphide silver method did not permit any differentiation between zinc iron and copper a granule fraction was isolated from cattle blood using glass re distilled water for haemolysis and fractionated centrifugation (*Josefsson & Paul to be published*) Ultrastructurally the granule fraction was more than 95 per cent pure with well preserved granules Atomic absorption spectrophotometry revealed

3 x 10 1f g zinc per granulum corresponding to ? 5 millions of atoms of zinc No copper or iron was found by this technique

Cattle eosinophilic granulocytes thus contain substantial quantities of zinc localized to specific granules Whether the metal is referable to metaldependent enzymes or has some yet unknown function remains to be investigated

L Grimelius A NEW ARGYROPHILIC STAINING TECHNIQUE FOR IDENTIFICATION OF THE α CELLS IN THE ISLES OF THE HUMAN PANCREAS

U Brunk & G Skold THE OXIDATION PROBLEM IN THE SULPHIDE SILVER METHOD FOR HISTOCHEMICAL DEMONSTRATION OF METALS

The metal content and its distribution in adrenal and liver tissue as shown with the sulphide silver method differs very much according to whether paraffin embedded tissue or cryostat se tions are used This can be shown to depend on oxidation probably to sulphites sulphates hydroxides and possible oxides of some of the metal sulphides during the process of deh dration and paraffin embedding The oxidation products cannot act as germs during the development. The sulphites and sulphates are later dissolved during staining By treating the sections with hydrogen sulphide gas before mounting and deparaffinisation most of the activity can be rest red but some diffusion artefacts cannot be prevented Because of this the use of cryostat sections is recommended Paraffin sections can be used only in cases dealing with metals the sulphides of which are resistant to oxidation

(Moberger HISTOCHEMICAL SULPHHYDRYL REACTIONS IN STUDIES OF CELL GROWTH AND CELL RIPENING

J Ericsson P Biberfeld & G H lm ELECTRON MICROSCOPIC STUDIES OF EXPERIMENTAL AUTO IMMUNE" NEPHROSIS

A nephrotic syndrome was induced in randomly bred female Sprague Dawley rats by 7 8 intraperitoneal injections of homologous kidney extract and Freund s adjuvant Portions of the renal cortex were fixed in OsO_4 and glutaraldehyde and the tissues were conventionally processed for light and electron microscopic studies Approximately 50 μ thick frozen sections of glutaraldehyde fixed tissues were incubated for the demonstration of acid phosphatase (AcPase) and were subsequently immersed in ammonium sulphide

Light microscopy of paraffin secti ns (aldehyde fixed t ssue) re eal d min r alterations in the renal co tex (possible slight thickening of gl merul r apill r walls "hyaline droplets in proximal tubules and occasional prot in us a t with dilatation of some po t ons of the nephron) Th n (appr ximat l 1 thi k sections of Epon embedd d glutaraldehyde fixed materi l incubated f r t c dem n stration of AcPase show d action product in many droplet —up to 4 n diamete —in the proximal con luted tubules other droplets—stainable w th t lu d ne b ue —did not acquire r t product de pite prolonged in ubat on

Electron micros j cale l lumpy bumpy deposits n the j th li l ide of the glomerular basem at m mbrane (bet veen the basement proper and th pithelial foot processes") an t h trophy of the epithelial cells The c toplasm of proximal tubule cells conta n l n merous electron dense "absorpt on dropl t and g eatl

increased numbers of large "cytosegresomes" and cytosomes. The latter appeared to carry acid phosphatase.

The observations indicate a focal damage to proximal tubule cells manifested by increased autophagocytosis. The localization of the deposits in the glomeruli on the epithelial side of the basement membrane may signify a non glomerular origin of the antigen(s) responsible for the immunologically induced renal disease.

I. Hammer, C. Nath rst Win lahl, S. J. Broberg. CORTISONE INDUCED NEPHROPATHIA IN RABBIT

C. Gustafsson, N. Chakravarty, S. J. Lahl. ULTRASTRUCTURAL CHANGES IN RAT MAST CELLS INDUCED BY ANTIGEN ANTIBODY REACTION AND COMPOUND 48/80

When a foreign protein together with pertussis bacilli is injected into rats, sensitizing antibodies develop in the body within 10–12 days and cause sensitization of mast cells. If such cells from the peritoneal cavity are exposed to the sensitizing protein, histamine is released and many cells disrupt. In order to follow the ultrastructural changes during this process sensitized mast cells were incubated with the homologous antigen and stained with Ruthenium red, an electron dense compound specifically reacting with and precipitating acid mucopolysaccharides. Histamine was determined using the biological method.

The most characteristic changes observed in the mast cells 5 minutes after the antigen antibody reaction were enlargement and diminished electron density of the central granules. Generally the peripheral granules lost contact with the extraplasm and looked relatively smaller and more electron dense. Complete dissolution of the granule with leaking of Ruthenium red positive material was rarely seen. When it occurred it was interpreted as a late and final stage. The synthetic compound 48/80 generally used to imitate anaphylactic histamine release produced similar changes in mast cells as the antigen antibody reaction. The observed changes were therefore considered to represent early and essential features in mast cell anaphylactic injury.

H. Helminen, J. Ericsson, S. Orrenius. ACID PHOSPHATASE ACTIVITY AND CELLULAR FINE STRUCTURE DURING ENFORCED MAMMARY GLAND INVOLUTION

Current theories concerning the pathogenesis of involution emphasize an active role of lysosomes. Suggested modes of action of lysosomes include (1) increased release of their enzymes with subsequent cell damage and lysis, (2) digestion in macrophages of previously damaged cells or cell constituents, and (3) focal cytoplasmic degradation in autophagic vacuoles.

In this study mammary gland involution was induced by removing the litter from lactating rats. Mammary glands excised during lactation or 1, 2, 3, 5, or 10 days after the induction of involution were studied by electron microscopy. Tissue was immersed in OsO_4 or perfusion fixation with glutaraldehyde (for the demonstration of acid phosphatase AcPase). AcPase was assayed biochemically in homogenates (total and nonsedimentable activity).

A rapid decline in gland weight, cell number and cell size occurred after the 3rd day. Significant alterations of the ratio nonsedimental to total activity of AcPase were not recorded. A moderate increase in specific AcPase activity was noted at 3

and 7 days Ultrastructurally numerous large cytogresomes—indicating auto or heterophagocytosis—were seen in the glandular cells during the whole involution period. Diffuse degeneration or dissolution of cells was not observed Sites of AcPase appeared larger and more numerous in glandular cells at 5 days and later and were confined to droplets (cytosomes and cytogresomes) Macrophages were not involved

These observations agree with the contention that the involution was attended by cytogresome formation Evidence for heterophagic digestion in macrophages or release of lysosomal enzymes was not obtained

O Hassler INCORPORATION OF THYMIDIN-H^3 IN THE BRAIN OF THE MOUSE AFTER A SINGLE X RAY DOSE

(Published in J Neur path Exp Neur 1 25 97 1966)

C Dallner THE FUNCTION OF THE GOLGI COMPLEX IN THE EXOCRINE PANCREAS

The active participation of the exocrine cell in the secretory process is reflected in a well developed endoplasmatic reticulum (ER) and Golgi complex Homogenization of guinea pig pancreas and subfractionation of the mitochondrial supernate in a sucrose gradient with cations gives 3 subfractions 1) rough microsomes (R) 2) smooth microsomes I (Sm I) and 3) smooth microsomes II (Sm II) In the electron microscope R consists of vesicles coated with ribosome Sm I of vesicles without attached ribosomes and Sm II of smooth vesicles and long paired membranes ver similar to the plasma membrane The possible identit of smooth vesicles with the Golgi complex is further indicated by incubating slices in the presence of leucin H^3 when a movement of the labelled protein from rough to smo th m crosomes occurs The maj rity of total protein and phospholipid (PLP) is in R (about 80 per cent) Sm I and Sm II contain more total cholesterol and triglycerides per PLP than R The *in vivo* incorporation rate of glycerol H^3 into lipid is significantly higher in the two smooth fractions in comparison with the rough one Acet l holin timulate inc rporation of P^3 into PLP of slices parti ularly in Sm I One electron tr nsport chain (DPNH Fpp x–cyt b ?) seems to be pres nt in total microsomes Anal i of the subfra tions reveals that DPNH–cyt c red activit i missing in Sm II TPNH–cyt c red is lower but ATPase and p–NPPase activities are higher in Sm I and Sm II in comparison with R Smooth vesicles wh ch most pr bably repre ent Golgi membrane exhibit diffe ences fr m the endopla mic reti ulum which i reco ered n f rm of rough microsomes The diffe ences can be related to pecial (l i fun t ons such as enz me transport and enz me concentrat on

S Jak bss n EFFECT OF EXPERIMENTAL DIABETES ON LIVER GLUCOSE PHOSPHATASE

In alloxan induced diabetes a change in the a[illegible] an in rea e in liver m r somal glucose–6–ph [illegible] to occur When allox n i injected to rat intr [illegible] acti ity sh w a r [illegible] ir rease and displ [illegible] glu ose tartrate or he ting demonst te th [illegible] of lys somal contam n t on Actinom in L [illegible] with all xan have n [illegible] at 5 hours b t [illegible] hours The

ultrastructure of liver cells shows a proliferation of smooth ER only later Smooth and rough microsomal subfractions exhibit a parallel increase of G6Pase activity in the first 5 hours A second rise at 10-15 hours however first appears in rough and later in smooth microsomes Three hours after alloxan injection K_m has changed from 2 5 to 4 9 (mM G6P) It remains at that level and is not influenced either by actinomycin or puromycin inhibitors of protein synthesis

Incubation of microsomes *in vitro* in the presence of 0 025 per cent alloxan elevates G6Pase activity and results in a K_m of 5 1 When the enzyme is solubilized alloxan has no effect on the V_{max} or the K_m

These results strongly suggest that alloxan in the liver influences primarily the membranes of the endoplasmic reticulum and thereby affects the kinetic properties of the enzyme These events precede *de novo* synthesis of G6Pase in the liver of alloxan diabetic rats

H Claumann PHOSPHOLIPIDS AND NEUTRAL LIPIDS ISOLATED FROM LIVER MICROSOMAL SUBFRACTIONS

Cation containing sucrose gradients are useful in subfractionations of liver microsomes In this way it is possible to obtain three subfractions 1) rough microsomes (R) 2) smooth microsomes I (Sm I) and 3) smooth microsomes II (Sm II) The lipids of these fractions were analysed The total microsomal fraction contains about 6 8 mg/g liver phospholipids (PLP) which corresponds to 85 per cent of the total lipids and 1 2 mg/g neutral lipids (NL) making up the remaining part The subfractions have different PLP/protein ratios but this difference disappears after a *washing procedure which removes the adsorbed protein and the luminal content* When the PLPs are separated on silicic acid impregnated paper the following distribution is obtained phosphatidyl (pH)-ethanolamin 18 per cent pH serine 9 per cent pH choline 48 per cent pH-inositol 11 per cent and sphingomyelin 5 per cent The individual PLPs display different rates of incorporation However the various subfractions exhibit the same patterns as well as specific activities upon comparison The incorporation rate of glycerol C^{14} into total PLP is very similar in R and Sm I but significantly slower in Sm II Separation of NL of total microsomes gives the following composition cholesterol (Ch) 0 6 mg/g cholesterol esters 0 07 mg triglyceride (TG) 0 5 mg free fatty acids 0 06 mg Ch is somewhat concentrated in Sm I but particularly in Sm II TG is twice as much in the smooth fractions as compared to the R *Measurement of total PLP after phenemal induced membrane synthesis* gives high values for R and Sm I On the other hand Sm II is constant and the absence of increase suggests a lack of participation in the process It is concluded that the membranes of the Sm I are in many aspects similar but not completely identical with those of the R Such a comparison results in an obvious difference in composition and turn over of membrane components of Sm II

O Hassler VASCULAR CHANGES IN SENILE BRAINS DEMONSTRATED WITH MICRO ARTERIOGRAPHY

Three kinds of cerebral vascular deformities i e glomerular loop formations, vascular *bundles and wickerworks* have been studied in 231 autopsied cases The changes were seldom observed in subjects below age 60 but were very common above age 70 They were almost equally distributed in the two sexes They were not particularly correlated with high heart weight high degree of cerebral arteriosclerosis

or low brain weight Vascular wickerworks were much more common in subject with vascular bundles than in those without The glomerular loops occurred as a rule together with marked spiralling and might be products of excessive spiralling

The influence of the vascular deformities upon the cerebral circulation was calculated partly on the basis of model experiments and partly by using *Poiseuille's* law Each glomerular loop may reduce the blood flow in the particular artery by about 60 per cent. The vascular bundles and wickerworks probably reduce the flow to about 10 per cent of the capacity of a single vessel of the same diameter Because the deformities only affect about one tenth of the arteries they probably do not influence the total cerebral blood flow more than the generalized spiralling This affects almost all arteries in cases with glomerular loops and probably reduces the total blood flow by about 10 per cent

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N *Ringert* ON CLASSIFICATION OF MAMMARY TUMOURS
SYMPOSIUM ON CELL PROLIFFRATION AND ITS CONTROL IN VITRO

K *Norrby* EXPONENTIAL HeLa CELL PROLIFERATION AS A FUNCTION OF RATES OF BIRTH DEATH AND GROWTH

Mathemati al estimators of proliferation of cell populations increasing in size have been based on comparatively simple models of proliferation Three factors may bias such estimator a) cell death b) a growth fraction less than one and in some systems c) a feeder compartment where a proliferative class is not self maintained but fed from s me stem cell class In tumour cell kinetics the si nificance of such a feeder factor is obscure Growth fraction may be determined by cinemicrographic analys s of a monola er culture Cell abortion may occur during exponential growth in a population but has n t been taken into consideration in mathematical estimators

The aim of this stud was to describe the growth of an e tablished cell l ne *in vitro* whilst taking int consideration the above mentioned bias particularl cell death. We have assumed that an exponential net cell increase in an as nchronou p pulation constitutes the difference between a more rapid exponential cell birth and an exponential and simultaneous cell death Acceptin th s assumption it i possible to deri e reasonably workable mathematical equations and we have thus mathematically defined rates of growth bi th and death These rates have been correlated to generation rates (reciprocal of gener tion times) r corded cinemicrographically in one and the same population and t ll mas n sister cultures

The rates of birth death and growth here presen ed give a atisfactory and v r tile description of the actual distribut on of l ıL † pa n te s in the cell populations

G *Moberg* r ON CLASSIFICATION OF TUMO RS OF THE URINARY BLADDER

Th Berge CARCINOMA METASTASES AND EXTRAMEDULLARY HAEMATOPOIESIS IN THE SPLEEN

In 181 cases with carcinoma metastases in the spleen a correlation was found between extramedullary haematopoiesis and the degree of carcinomatous bone marrow replacement. But factors other than bone metastases are of importance and an increased frequency of extramedullary haematopoiesis was found only in cases with pronounced replacement of bone marrow (cancer involving all vertebrae). In cases in which some of the vertebrae only are involved extramedullary haematopoiesis was found to proceed at the same rate as in cases without skeletal engagement. The theories that tumour growth in the spleen might stimulate extramedullary haematopoiesis or vice versa were not supported by the investigation.

I Angervall, B Stener, C I Wahlström CLEAR CELL SARCOMA OF TENDONS AND APONEUROSES

Two cases of clear cell sarcoma of tendons and aponeuroses were described, one of these apparently originating from the tendon of vastus medialis (28 year old man), the other from the tendon of tibialis anterior (60 year old man). This type of sarcoma was described by *Enzinger* (1965) as a new tumour entity. The tumour is characterized by pale round or fusiform cells of epithelioid appearance arranged in compact nests and fascicle. The nuclei are pale staining but have a prominent nucleolus. Mitotic figures are scarce. The cellular aggregates are enclosed by delicate septa of fibrous connective tissue. These septa are continuous with dense collagenous structures that transverse the tumour and merge with preformed tendons and aponeurose.

The most difficult differential diagnosis is synovial sarcoma. An important point of distinction is the total lack of biphasic differentiation in clear cell sarcoma. The tumour also differs from synovial sarcoma as to the content of mucopolysaccharide and its clinical course is more protracted. Thus even though the ultimate prognosis is grave, the 5 year survival rate after operative treatment is high.

K Norrby, I Enerbäck, C L Schelin DIFFERENTIAL DIAGNOSIS OF OVARIAN NEOPLASMS SIMULATING MALIGNANT BRENNER TUMOURS

Two cases of malignant ovarian neoplasms were reported, both interpreted as primary ovarian tumours at operation. Later subileus due to tumour masses in the mesentery and the small intestine developed in both patients. They died 10 and 17 months respectively after the first operation, exhibiting distant metastases.

Both ovarian tumours were solid and were of a similar morphological appearance with considerable cellular atypia. They were interpreted as possibly malignant Brenner tumours. The development of intestinal tumours led to a reconsideration of the diagnosis utilizing histochemical methods.

The tumour cells in formaldehyde fixed specimens from one of the cases showed histochemical properties strongly indicating a content of hydroxytryptamine (serotonin). Thus the tumour cells displayed reducing properties, alkaline diazo coupling, colour reaction with ninhydrin and fluorescence in ultraviolet light. In addition the presence of 5-hydroxytryptamine was demonstrated by means of the borhydride technique (*Enerbäck*, to be published). All the histochemical reactions were negative in the other case which thus might represent a malignant Brenner tumour.

The findings clearly demonstrate the value of hist chemical reacti ons in th diag n sis of ovarian tumours simulating Brenner tumours and call attentic n to carci noid as a diagn stic alternative in these case

Chr Ahren L Angervall B Stener & I Stener OSSIFYING PSEUDO TUMOURS IN SOFT TISSUE

I S urander & Y Olsson MORPHOLOGICAL AND LIPID HISTOCHEMICAL STUDIES ON PERIPHERAL NERVES IN GLOBOID CELL LEUCODYSTROPHY (Mb KRABBE)

It is generally believed that peripheral nerve remain unchanged in gl loid cell leuco dystrophy (GLD) Thi opinion seems to be based on rej orted findings in only four cases of GLD in which peripheral nerves were said t be normal But since the methods used and the examined regions of the perip heral nervous system were not stated the involvement of perij he al nerves in GLD must be considered an unso ved question

We have examined vari us levels of the peripheral nervous system in six clinicall and hist logi ally typical cases of GLD All the peripheral nerves (sciatic femoral sural and cutaneo u nerves of the calf) pre ented severe degenerati n of axons and myelin sheaths end neurial fibro is and periva cular ac umulations of histiocyte Spinal nerve r ots and d rsal root ganglions were als severely damaged Lipid histochemical studie f m elin bre kdo n products in peripheral nerves from tw cases revealed the accumulation of a PAS positive lipid m st prol ably cerebroside in S hw ann cells and endon urial phagocytes A pre ence of sudanoj hilic material in the nerves was not observed

In Wallerian degenerat on the myelin lipids including cerel roside are r moved nd sud nophilic cholesterol esters are formed Thus the lipid histochemical pat te n in our two cases of GLD is not of the Wallerian t pe but is most prcbably directly related to the underl ing metab lic defect in GLD

I Fnerba k ACTION OF SODIUM BOROHYDRIDE ON THE CHROMOGENIC MATERIAL IN CARCINOID TUMOURS

S dium bo oh dride as a reducing eagent in the histochem try of m noamine w s intr duced by *C rr li Hilla p & Jonss n* ho u ed t a a means by which the sj ecificity of the formald hyd gas method might be incre ed Preliminary data n the effect of sodium b h dride and formaldehyde g n the hr mog n material in arcinoid tum ur fixed in formalin sol iti r p t

The yellow fluores en urring in the tumour ce l l rr l tin *gui hed by* bor hydrid t tm nt and partly rege er *L* o q i to m dehyd g s treatment

A modified ninhyd i d re ulted in an o n t c in th tum ur cells in a ldit n t h orange colour de l *H l* *t l en utt* Enter chromaffin hibited a red fluores a l lou n pe m ns fixed in f r l n t on but n t in freez f m ld hyde g treated specimens he n dr n induced flu es un ffected i hydri l tr atment

The find ngs sug t th e cl romogen mate ial in h d ox tr j m n taining structure xed n i m l n soluti n m y d ffer fr m th t n fre z dried and formaldeh l a t t tis ue

On the basis of these findings a new technique for the demonstration of 5 hydroxytryptamine in formalin fixed tissues is proposed This technique utilizes the effect of borohydride and subsequent formaldehyde gas treatment on the yellow autofluorescence and the orange red fluorescence induced by ninhydrin and seems to offer a higher degree of specificity than that obtainable by methods hitherto available

B Boeryd B Hagmar C C Johnsson EFFECT OF HEPARIN EPSILON AMINO CAPROIC ACID AND GUANETHIDINE ON INTRAVENOUSLY INDUCED METASTASES OF MELANOMA B16

In previous studies on the distribution and growth of intravenously induced metastases EACA increased the average and total metastasis volumes in the first capillary bed the lungs in two of three isologous systems Apart from its antifibrinolytic effect EACA affects catecholamine metabolism It shares this effect with guanethidine in which known antifibrinolytic activity is lacking

It might be possible to evaluate the relative importance of the antifibrinolytic and sympathetic blocking properties of EACA by comparing the effects of EACA and guanethidine on metastasis formation

Experiments were therefore performed on inbred C57BL/6J mice with an isologous tumour Melanoma B16 An enzymatic tumour cell suspension was inoculated intravenously into controls heparin EACA and guanethidine treated mice

In EACA treated as well as in guanethidine treated mice the number and total volume of metastases to the lungs increased while the average metastasis volume remained unchanged Heparin decreased the average and total metastasis volumes to the lungs and the number tended to be reduced

EACA and guanethidine had similar promoting effects on pulmonary metastases Both drugs deplete the catecholamine stores Presumably this effect somehow contributed to the increased number of pulmonary metastases after administration of EACA

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SUBEPITHELIAL CHANGES IN ORAL SUBMUCOUS FIBROSIS

By

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Received 9 xi 66

Submucous fibrosis was *reported as a distinct morbid entity of the* human oral mucosa by *Joshi* (1953) He described the symptoms as vesicles on the palate in early cases gradually increasing oral fibrosis *leading to trismus and a marked inability to eat food spiced* with chillies The identical oral state has been called variously as Atrophia idiopathica tropica mucosae oris (*Schwarz* 1952) "Diffuse oral submucous fibrosis (*Lal* 1953) idiopathic scleroderma of the mouth" (*Su* 1954) Idiopathic palatal fibrosis (*Rao* 1962) and "Sclerosing stomatitis (*Behl* 1962)—the interesting point being that except for *Su*s cases which occurred in the Chinese in Taiwan this condition has been reported only in Indians Clinical aspects of the disease its histopathology and probable etiology have all been commented upon during the last few years (*Sirsat & Khanolkar* 1957 1960 1960a 1960b 1962 1962a *DeSa* 1957 *George* 1958 *Sharan* 1959 *Pindborg et al* 1964) A *recent paper reports the epithelial changes that occur in the oral* mucosa in submucous fibrosis (*Pindborg et al* 1965) This paper describes in detail the subepithelial changes that supervene in submucous fibrosis and assays a histological comparison in early and advanced stages of the disease

MATERIAL AND METHODS

This stud is based on biopsies obtained from two series of patients One group of 8 cases was obtained from private or hospital ear nose and throat linics n Bombay or from the admission clin cs of the Tata Memorial H spital, Bombay The second group of 114 cases was collected from the Go t. Dental Colleg n Lucknow Bombay and Trivandrum Mo t of the tissues we e punch on biop ies from fibrotic areas on the bu al mucosa Occasionally an add t nal b p was taken from the palate gingiva or lip Fixation was in 10 per er n a bu fer d forma

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Supported by grant f m the *Danish State Resea ch F undati n* and 4 *vid Nils s ns Foundation* On tnor (SMS) also recei ed inan al p t fr m the *World Health Organi ati n*

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5 μ thick serial sections were stained routinely with haematoxylin eosin Mallory's trichome (Weinmann & Meyer 1959) Cridley 1957) Mallory's phosphotungstic acid haematoxylin Weigerts resorcin fuchsin (Harts modification) and toluidine blue at pH 4 5

RESULTS

On the basis of the histopathology in H & E sections the biopsies from submucous fibrosis can be grouped into four clearly definable stages 1) very early 2) early 3) moderately advanced and 4) advanced These stages have not been judged only on the amounts and nature of the subepithelial collagen but on the following criteria in combination 1) presence or absence of oedema 2) physical state of the mucosal collagen 3) overall fibroplastic response (number of cells and age of individual cells) 4) dynamic state of blood vessels and 5) predominant cell type in the inflammatory exudate

The four stages have been described as follows

1) *Very early*

Collagen finely fibrillar dispersed by the presence of marked oedema Fibroblastic response strong with plump young cells with abundant cytoplasm Blood vessels sometimes normal often dilated and congested Inflammatory cells mainly polymorphonuclear leucocytes with an occasional eosinophil (Fig 1)

2) *Early*

Juxtaepithelial area shows early hyalinisation Collagen still seen as separate bundles which are thickened (this term is used to denote early coalescence within the bundle—which is still recognisable as a separate wavy unit) Plump young fibroblasts are present in moderate numbers The blood vessels are often dilated and congested The inflammatory cells are mostly mononuclear lymphocytes eosinophils and an occasional plasma cell (Fig 2)

3) *Moderately advanced*

Collagen is moderately hyalinised the amorphous change starting from the juxtaepithelial basement membrane Occasionally thickened collagen bundles are still seen separated by slight residual oedema The fibroplastic response is much less the cells present being mostly adult fibrocytes with elongated spindle shaped nuclei and scanty cytoplasm Blood vessels are either normal or constricted due to increased surrounding fibrous tissue The inflammatory exudate consists mainly of lymphocytes and plasma cells though an occasional eosinophil is seen (Fig 3)



Fig 1 V ry ea l tage of l sulmucosa fibr s k d ascul r dilatati n oedema an nflamm n are present Haemat xylin eosin × 155

Fig 2 Early stag of oral bmucou fibrosi Ju xtaepithel l hyal ni at n v s cular dila tion oed n i inflammat ry cells nd plump fibrobla t r present. H motox li osin × 370



Fig 3 Moderately advanced oral submucous fibrosis. Moderate hyalinisation of mucosal collagen; separate bundles still seen; fibroblasts residual and thin. Blood vessels narrowed. Haematoxylin eosin × 370

Fig 4 Markedly advanced stage of oral submucous fibrosis. Mucosal collagen completely hyalinised, mostly acellular; few vestigial fibroblastic profiles are seen. Blood vessels obliterated. Haematoxylin eosin × 370

4) *Advanced*

Collagen is completely hyalinised and is seen as a smooth sheet with no separate bundles discernible Oedema is absent The hyalinised areas are devoid of fibroblasts though a thin elongated cell or vestigial nucleus is seen at rare intervals along the fibre bundle Blood vessels are completely obliterated or narrowed The inflammatory cells are lymphocytes and plasma cells (Fig 4)

Table 1 shows the number of biopsies in the different stages of sub epithelial response

TABLE 1

Summary of Subepithelial Response in 199 Cases of Oral Submucous Fibrosis

Investigators	Place	Total No of cases	Stage of subepithelial response			
			Very early	Early	Moderately ad vanced	Ad vanced
Pindborg et al	Lucknow	32	1	20	8	3
Pindborg et al	Bombay	42	5	17	15	4
Pindborg et al	Trivandrum	40	1	17	17	5
Sirsat & Khanolkar	Bombay	85	–	30	36	19

The following Indian doctors co operated in collecting the material *T N Chawla D Gupta H K Kalapessi S A Kale B Singh A N Srivastava B N Talyarkhan* and *J Zachariah*

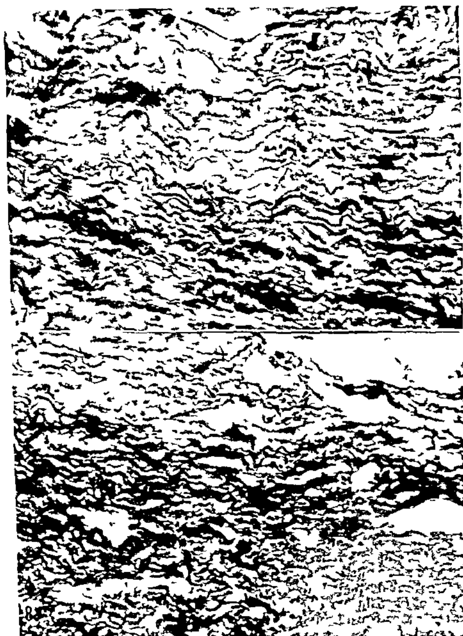
The fibrous connective tissue has been studied by three differential stains—Mallorys trichrome Mallorys phosphotungstic acid haemat oxylin (PTAH) and Weigerts resorcin fuchsin The tinctorial changes of the collagen in submucous fibrosis as seen in serial sections have been evaluated on the basis of combined reaction to all the three stains Described in sequence of severity of alteration the different patterns of response are as follows

1) Dense thickened collagen staining evenly blue and evenly orange with the two Mallory methods and negatively with resorcin fuchsin
2) Uneven pale to intense staining with the two Mallory techniques negative to resorcin fuchsin
3) Chromophobic or pale staining areas with the two Mallory stains (Fig 5) with identical area in serial section filled with resorcin positive thickened coarse fibres bits granules (Fig 6)
4) Identical fibre bundles partially or full yellow or yellow red with the trichrome partially or fully purple with PTAH (Fig 7) and with mild moderate or massive amounts of resorcin positive material of the altered fibre bundles (Fig 8)
5) *Coalesced hyaline masses staining yellow with the trichrome* purple with the PTAH and positive with resorcin (Fig 9 10 11 12)



Figs 5 and 6

Subepithelial collagen showing chromophobia with PTAH (Fig 5) and marked increase in resorcin positive fibrous material in identical area in serial section (Fig 6)
Fig 5—Mallory's PTAH × 360 Fig 6 Weigert's resorcin fuchsin × 360



Figs 7 and 8

Subepithelial coll[illegible] wing uneven pale to int[illegible] st[illegible] crys[illegible] chrome (Fig 7) [illegible] rein positive s[illegible] f bres in serial section Fi[illegible] — [illegible]lor's trichrome × 360 F[illegible] — We[illegible]rt [illegible] fu hsin × 360

This basic tinctorial alteration has been qualitatively classified as mild, moderate or massive alteration according to how much total area in the section shows tinctorial change. Table 2 shows the number of biopsies in each group in relation to the histological grading seen in H & E sections.

TABLE 2

Reaction of Subepithelial Collagen to Differential Stains for Connective Tissue in Submucous Fibrosis of the Oral Mucosa

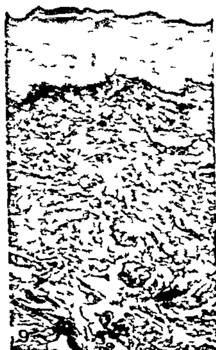
Histological stage	Tinctorial reaction of collagen to differential stains for connective tissue				
	No alteration in staining	Uneven staining no altered	Altered staining in occasional bundle	Altered staining in many bundles	Massive altered staining
Very early	–	–	1	3	–
Early	3	15	17	23	4
Moderately advanced	–	6	11	18	11
Advanced	–	–	2	5	13

DISCUSSION

Table 1 lists the biopsies in the four histological stages—very early, early, moderately advanced and advanced. In the first group of 114 cases collected by *Pindborg* and his co-workers, 7 show very early and 54 show early changes. Fifty-two are in the moderately advanced or advanced group. In the cases studied a few years earlier by *Sirsat & Khanolkar* (1962) there are no biopsies showing a very early response, 30 show early changes, while as many as 55 of the 85 are moderately or markedly advanced. This marked difference in the grade of subepithelial response in the two series is probably due to two reasons. The criteria used to recognise the disease in the 85 cases in the first group were marked fibrosis or trismus. A majority of biopsies in this group were obtained from ear, nose and throat clinics where the patient came to seek relief from the acute distress caused by the clinical complications of submucous fibrosis. The second group of 114 biopsies was collected from dental clinics, and the presence of a blanched mucosa or palpable fibrous bands even in clinically asymptomatic patients was considered sufficient indication of the disease to take a biopsy. It is not surprising, therefore, that with such different clinical

Figs 9, 10, 11 and 12

Eosinophilic hyaline masses (Fig. 10) staining well with Mallory's trichrome (Fig. 9), purple with Mallory's PTAH (Fig. 12) and showing resorcin-positive material in identical area in serial section (Fig. 11). Fig. 9—Haematoxylin-eosin × 125. Fig. 10—Mallory's trichrome × 125. Fig. 11—Mallory's PTAH × 125. Fig. 12—Weigert's resorcin fuchsin × 125.


9


10


11


12

base lines *Sirsat & Khanolkar's* 85 cases and the cases collected by *Pindborg* and his collegues show such a marked difference in the distribution of histological stages of response

In 34 cases more than one biopsy was taken from clinically affected areas on the same buccal mucosa An analysis of the histological grading in the multiple biopsies shows that in 22 patients both or all three biopsies showed a similar severity of histological response In 12 patients however the biopsies from different areas showed difference in stages of histological response early in one tissue and moderately or markedly advanced in the other This can be explained on the basis of focal initiation of the disease process in the mouth and therefore a natural unevenness in severity of the subepithelial change in different parts of the mucosa

Normal collagen stains an even blue and even orange with the two Mallory stains It is negative to resorcin which usually demonstrates the other fibrous scleroprotein of connective tissue elastin In this study 3 biopsies, while showing densely thickened collagen typical to this disease stained normally and 21 showed uneven staining especially in the deeper parts of the mucosa but with no abrupt tinctorial change While this uneven staining is probable the earliest sign of collagen alteration it has not been considered as much as it is found though very occasionally in tissue believed to be normal On the basis of altered staining reactions in submucous fibrosis the initial temptation is to suggest frank fibrinoid alteration of the submucosal collagen Recent work on the immunohistochemistry of fibrinoid denotes the presence of a number of diverse vascular and sensitivity mechanisms (*Gitlin et al* 1957 *Vazquez & Dixon* 1937 1958) Knowledge of the primary patterns of tissue response in submucous fibrosis is still too meagre to take for granted any such physiological sequence of events *Unna* (1896) has described staining changes similar to those seen in submucous fibrosis in aging or degenerating collagen and called this altered material collastin More recently *Gillman* (1955) and his group have studied altered collagen in skin and vascular endothelium and also find abnormal staining similar to that seen in submucous fibrosis They call this marked transformation pseudoelastic or "elastotic" degeneration Till the alterations seen in submucous fibrosis have been characterised more minutely at molecular levels with the sophisticated techniques available today this histochemically descriptive term serves the purpose well It is also in keeping with the submicroscopic degradation of altered collagen into structures similar to the dual forms of elastin (*Sirsat* 1958 *Sirsat & Khanolkar* 1960) and its susceptibility to digestion with crude elastase (*Sirsat & Khanolkar* 1960) Age of the host in relation to the amounts of tinctorial alteration seen in the oral mucosa collagen is an important parameter to consider in evaluating changes specific to the disease Table 3 shows the amounts of change seen plotted against the ages in decade of patients There is

no selective correlation between the age of the patient and the amounts of tinctorial alteration in collagen with the differential stains for connective tissue

The subepithelial reaction seen in submucous fibrosis is a continuous chronic inflammation and residual oedema with simultaneous attempts at repair with fibrosis *This coexistence of a defense as well as reparatory mechanism is termed a chronic productive response (Payling Wright 1954)* The conditions under which a non viable agent can excite this response are when its action may be mild or its concentration too low to produce an immediate severe injury Such an agent then operates more or less continuously for a long period with only moderate *severity and the resulting tissue reaction* assumes a more complicated pattern than is found with either acute inflammation or with tissue repair alone (*Payling Wright* 1954) When the nutritional state of the mucous membrane is defective the local defences of the host are retarded and any mild injury instead of ending in rapid uneventful healing may result in chronic inflammation In moderately or markedly advanced biopsies of submucous fibrosis the blood vessels show a narrowed or blocked lumen due to increasing pressure of dense fibrous tissue(*Sirsat & Pindborg* 1967a) This undoubtedly results in impaired nutrition to all the oral mucosal elements This point as also the over all malnutrition of the host system in the poor Indian patient has to be borne in mind when assessing the persistent mild to moderate chronic inflammation found in a majority of biopsies

TABLE 3

Summary of Correlation between Age of Patient and Altered Collagen in [illegible] Biopsies of Oral Submucous Fibrosis

Degree of change in collagen	Age (in years) 21 30	31–40	41 50	51–60	61–70	71–80	Total Biopsies
No change	2	1	–	–	–	–	3
Uneven staining	5	4	4	6	2	–	21
Altered staining in occasional bundle	3	9	7	10	3	–	32
Altered staining in many bundles	4	7	20	10	6	1	48
Massively altered staining	5	5	8	2			[illegible]

The distribution of cell types in the inflammatory exudat [illegible] and advanced biopsies raises a few interesting point related [illegible] functional relationship of the leucocyte to the r[illegible] In very early and [illegible] cases the main cell [illegible] polymorphonuclear neutrophil The emigration of th[illegible] from th[illegible] blood stream into tissu[illegible] spaces is an established [illegible] in non immune acute in[illegible]flammation in man However with [illegible] chron

ients of tissue response and with the probable operation of other indirect systemic factors there is an increase in tissue eosinophils which remain in residual numbers even in moderately advanced biopsies In the moderately or markedly advanced cases the predominant inflammatory cells are lymphocytes and plasma cells The lymphocyte is believed to have a multipotential role in a trephocytic storage of protein (*Kelsall & Crabb* 1958) in fibroplasia through transformation into fibroblasts (*Dumont* 1959) and in the probable production of tissue immunity (*Gowans* 1962) While these interrelationships still merit further experiment the parallel presence of large numbers of lymphocytes and fibroblasts as well as plasma cells in moderate numbers emphasise the importance of sustained lymphocytosis in the maintenance of the tissue reaction in submucous fibrosis Early and persistant tissue eosinophilia as also the presence of plasma cells also make it worth while to investigate further the earlier suggestion that the pathogenesis of submucous fibrosis of the oral mucosa might lie in a mild immune response (*Sirsat* 1958 *Pindborg & Singh* 1965) Preliminary studies on this basic facet of the disease are in progress

SUMMARY

One hundred and ninety nine biopsies of oral submucous fibrosis have been studied by haematoxylin eosin and differential stains for connective tissue The disease can be classified into very early early moderately advanced and advanced stages on the basis of the histological response The nature of connective tissue changes the persistance of mild to moderate chronic inflammation and the distribution of cell types in inflammatory exudate make it worth while to investigate an earlier suggestion that the pathogenesis of oral submucous fibrosis might be in a mild immune response

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MAST CELL RESPONSE IN EARLY AND ADVANCED ORAL SUBMUCOUS FIBROSIS

By

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Received 2 xii 66

The clinical and pathological features of oral submucous fibrosis have been described by a number of workers (*Joshi* 1953 *Lal* 1953 *Su* 1953 *Sirsat & Khanolkar* 1957 1960 1962 *De Sa* 1957 *Rao* 1962 *Pindborg & Singh* 1964 *Pindborg & Zachariah* 1965) Recent reports show the disease is fairly wide spread in all parts of India (*Pindborg & Sirsat* 1966) A significant correlation between the parallel presence of submucous fibrosis and cancers of various sites in the oral mucosa has also been noted (*Paymaster* 1957 *Pindborg & Zachariah* 1965) The aetiology of this condition remains obscure though the slow irritant action of capsaicin (the irritant principle of chillies—Capsicum annum) has been assessed on the rat oral mucosa as a possible causative factor (*Sirsat & Khanolkar* 1960a 1960b) The subepithelial response in oral submucous fibrosis (*Sirsat & Pindborg* 1967) as also in the vesicles which occasionally erupt as an early clinical sign (*Pindborg & Singh* 1965) suggest a rather complex pathogenesis Biopsies from patients with this disease are therefore being studied from various aspects towards understanding the basic reactions in it This paper reports the mast cell response in histologically early and advanced stages of the disease

MATERIAL AND METHODS

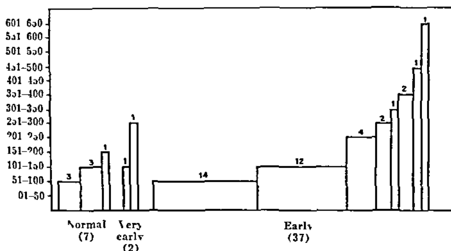
Biopsies from 80 cases of submucous fibrosis were available for this study The origin of the biopsies the manner in which they were obtained and processed have all been described in detail (*Sirsat & Pindborg* 1967) 6 μ thick paraffin sections were stained with haematoxylin and eosin to establish the diagnosis and histological stage of the disease Serial sections were stained with 1 per cent toluidine blue at pH 4 5 The mast cell counts were carried out on each section in one hundred 10×45 microscopic fields Great care was always taken to maintain uniformity in counting starting from the right upper edge of the section in every ca

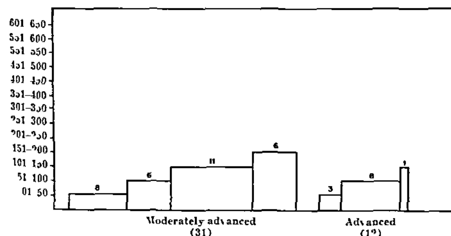
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Mast cell response in 80 biopsies of oral submucous fibrosis
(figures in brackets indicate total cases in each histological stage)

RESULTS

An earlier paper (*Sirsat & Pindborg* 1967) grades the subepithelial response in submucous fibrosis into four stages 1) Very early 2) Early 3) Moderately advanced and 4) Advanced Fig 1 shows the mast cell counts in 7 samples of normal buccal mucosas 2 very early 37 early 31 moderately advanced and 12 advanced biopsies The range seen in the seven normal oral tissues is from 51–100 to 151–200 Of the two very early cases one shows a count of 101–150 and the other between 251–300 In the 37 early cases twenty six are within the range shown by the normal tissues Nine have mast cell counts from 201–250 to 351–400 Two biopsies show a massive increase in mast cells one in

451–500 group and the other in the 601–650 group The moderately advanced biopsies all show mast cell counts either similar to normal or as in 8 biopsies even lower than in normal tissue Similarly so in the advanced cases where out of 12 cases three are 1–50 8 in the 51–100 group and one in the 101–150 group

DISCUSSION

The ideal human buccal mucosa for assessing normality would be from persons in whom no trauma ever occurred That however is not such a practicable event as apart from the common adult habits of smoking and chewing tobacco or the lime—betel mixture in the East the oral mucosa is exposed to sudden variations in temperature and repeated injury during mastication The seven biopsies taken from buccal mucosas apparently free from disease show a range in mast cell counts from 51–100 to 151–200 This range has been considered normal for purposes of comparison

The qualitative aspect of mast cells usually described is either the structural integrity with retention of the metachromatic granules or partial or complete degranulation of the cell The significance of loss of granules from the mastocyte cytoplasm is still a matter of debate Some workers (*Devitt et al* 1940) believe that mast cell degranulation is a sure morphological indicator of histamine release Others feel that this phenomenon is a non specific one brought about by many factors (*Selye* 1965) including hydration and mechanical injury (*Fawcett* 1955) Degranulation or its absence has not been commented upon in this study In counting partially degranulated cells obviously mastocytes were also included An interesting observation was that in a majority of sections classified histologically as early with much vascular dilatation and oedema the mast cells were mainly perivascular In the moderately advanced and advanced tissues the few cells found were scattered randomly in the tissue

The pattern of mast cell response in oral submucous fibrosis is consistent with the overall sequential tissue response to the disease In the early cases where reaction of the tissue to the irritant is the strongest the mast cell counts are highest As the tissue gets converted to less reactive hyaline occasionally degenerating mass the mast cells become less and are often even fewer than in the normal mucosa It is interesting to compare this response to that seen in inflammation There is general agreement that in acute inflammation there is a decrease in tissue mast cells (*Lever* 1961) In the chronic state where there are cyclic incidents of necrosis or alternating proliferative and degenerative changes the mast cells counts are higher than those in normal tissue According to *Mc Cutcheon* (1953) as chronicity increases vascularity and oedema decrease while mast cells increase in number In oral submucous fibrosis as the disease advances there

is a drop in the number of mast cells being even fewer than found in the normal buccal mucosa This is probably so because as the morbidity progresses the actively vascular oedematous connective tissue alters gradually to a smooth inert hyaline mass which does not encourage the maintenance of cell life Active degranulation of the mast cells with continuous histamine release in the early stage might also account for the paucity of mast cells in the more advanced stages of the disease Vascular dilatation and persistent oedema are predominant features of early stages of submucous fibrosis These symptoms along with the high mast cell counts are tissue reactions similar to those described in certain mesenchymoses believed to be of autoimmune origin (*Asboe Hansen* 1964) If there is a trigger substance which elicits the marked increase in this histamine synthesising cell in sensitive human subjects the need to recognize it is very clear The mast cell response in relation to the overall subepithelial response (*Sirsat & Pindborg* 1967) the presence of circulating and tissue eosinophilia (*De Sa* 1957 *Rao* 1962 *Pindborg & Singh* 1965) and the gammaglobulinaemia reported by some authors (*De Sa* 1957) all hint at the possibility that the pathogenesis of oral submucous fibrosis might lie in the realm of immunopathology Further investigations are in progress in order to decide whether this is really so

SUMMARY

Mast cell counts were carried out on toluidine blue stained sections in 80 biopsies of oral submucous fibrosis and 7 normal control buccal mucosas One hundred 10×45 microscopic fields were counted in each section In the early cases 9 out of 37 biopsies showed a significant rise in the mast cell population In the very early moderately advanced cases they were similar to or less than those seen in the normal mucosa The results are discussed from the viewpoint of the probable pathogenetic mechanism of the disease

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THE VASCULAR RESPONSE IN EARLY AND ADVANCED ORAL SUBMUCOUS FIBROSIS

By

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Oral submucous fibrosis has been recognised as a distinct clinical and pathological entity (*Sirsat & Khanolkar* 1962 *Pindborg & Sirsat* 1966) The etiology and pathogenesis of this disease have not been fully understood as yet Attempts to do so are being made by studying the basic tissue reactions in this oral disorder This paper reports the vascular response in early and advanced stages of the disease

MATERIAL AND METHODS

One hundred and twenty four biopsies were available for this study from patients in which a clinical and histological diagnosis of oral submucous fibrosis had already been made Serial sections were stained with haematoxylin and eosin to establish the histological stage of the disease and Weigerts resorcin fuchsin for elastin (Hart s Modification) to observe the vascular elastic fibres

RESULTS

The criteria used to grade the tissue response in oral submucous fibrosis have been described in detail (*Sirsat & Pindborg* 1967) In the biopsies studied 4 were in the very early stage 61 in the early stage 50 in a moderately advanced and 9 in an advanced stage Among the bases used to grade the histological stage of the disease was the state of the blood vessels which appeared to show extreme dilatation in the early stages and narrowing in the more advanced stages A detailed study shows however that the pattern of blood vessel reactions is more complicated than initially thought of and normal dilated and constricted vessels are found in the same section (Figs 1–4) The response has been therefore assessed in six groups according to the type of single or combined blood vessel reaction present These groups are normal normal.dilated dilated dilated.constricted normal.constricted and constricted The accompanying histogram (Fig 5) shows the pattern of response in the 124 sections of submucous fibrosis observed In the thirteen

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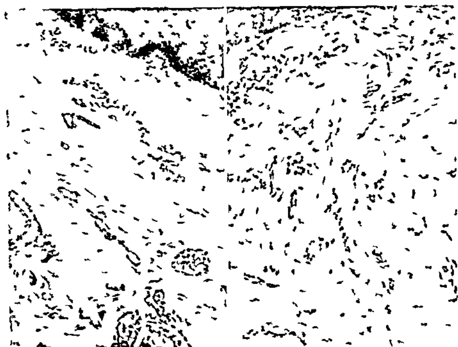


Fig 3　　*Fig 4*

Fig 3 Section from mucosa in oral submucous fibrosis showing hyalinised connective tissue The blood vessels in the upper mucosa show constriction Those in the deeper layer are dilated some exhibiting stasis H & E. × 115

Fig 4 Section from mucosa in oral submucous fibrosis There is marked constriction of the lumen in all blood vessels some showing complete obliteration of lumen *H & E. × 115*

This could be either due to the presence of mild subclinical inflammation or a greater sensitivity of these two subjects to local infiltration with anaesthetic prior to biopsy In none of the 13 was there any constriction of the blood vessels seen—though one showed a mild hyalinisation of the connective tissue

The microvascular aspects of tissue injury with increased permeability of the endothelium and dilatation of the vessels accompanying inflammation are only too well known In oral submucous fibrosis inflammation is very commonly found coexistant to the connective tissue repair processes It was necessary therefore to analyse the vascular response in terms of the inflammatory exudate present Table 1 shows the types of vascular reactions seen in the 124 biopsies where inflammation is absent minimal mild moderate marked or massive In 14 biopsies where inflammation has subsided completely one shows a persistant dilatation of all blood vessels [illegible] 8 shows dilatation of a few vessels and a constricted ones [illegible] a normal constricted reaction while in two all vessels are in various stages of constriction Of th

COMPARISON OF VASCULAR RESPONSE IN RELATION TO HISTOLOGICAL STAGE OF ORAL SUBMUCOUS FIBROSIS

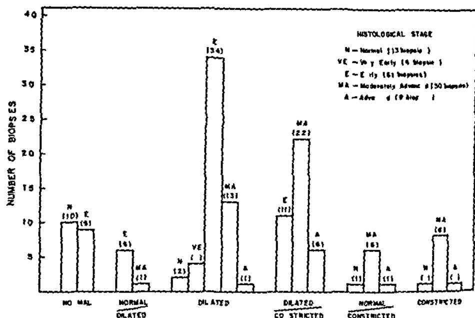


Fig 5

Histogram showing comparison of vascular response in relation to histological stage of oral submucous fibrosis

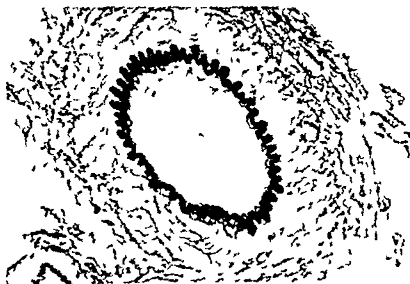


Fig 6

Section from mucosa in oral submucous fibrosis showing a deeper arteriole with a healthy vascular elastica Weigerts resorcin fuchsin × 11

TABLE 1

Summary of Vascular Response in Relation to Inflammation in 125 Biopsies of Oral Submucous Fibrosis

Histological stage	Inflammatory exudate	Number of biopsies	Vascular response: Normal	Normal/dilated	Dilated	Dilated/constricted	Normal/constricted	Constricted
Very early (4)	None	–	–	–	–	–	–	–
	Minimal	–	–	–	–	–	–	–
	Mild	–	–	–	–	–	–	–
	Moderate	4	–	–	4	–	–	–
	Marked	–	–	–	–	–	–	–
	Massive	–	–	–	–	–	–	–
Early (61)	None	3	1	1	–	–	–	1
	Minimal	3	2	–	–	1	–	–
	Mild	25	5	5	12	3	–	–
	Moderate	13	1	–	8	4	–	–
	Marked	10	–	–	8	2	–	–
	Massive	7	–	–	6	1	–	–
Moderately advanced (50)	None	9	–	–	1	6	1	1
	Minimal	4	–	–	–	3	–	1
	Mild	16	–	–	2	5	3	6
	Moderate	18	–	1	9	7	1	–
	Marked	3	–	–	1	1	1	–
	Massive	–	–	–	–	–	–	–
Advanced (9)	None	2	–	–	–	2	–	–
	Minimal	1	–	–	–	–	–	1
	Mild	4	–	–	–	3	1	–
	Moderate	–	–	–	–	–	–	–
	Marked	2	–	–	1	1	–	–
	Massive	–	–	–	–	–	–	–

45 biopsies which had mild inflammation 14 had a marked dilatation of all blood vessels while 21 had a few or all vessels with narrowed lumen. In the 57 biopsies showing moderate to massive inflammation 46 showed massive dilatation following the natural sequence of events in a tissue reaction to trauma. The remaining 18 biopsies had a number of vessels in various stages of narrowing which was apparent first in the smaller vessels in the upper mucosa and spread gradually to the larger deeper vessels. This follows the pattern of hyalinisation in this disease starting initially in the juxtaepithelial zone and spreading downwards with increasing intensity of the morbid process. The constriction of the mucosal blood vessels is therefore an obvious result of the increasing pressure exerted by the densely fibrous or frankly hyaline environment. The significance of the impaired nutrition to the tissue due to the limited blood supply lies in its effect on the overlying mucosal epithelium. The lack of optimum metabolic precursers could be responsible for the aberrent restless epithelium seen occasionally

and the frankly atypical one seen more uncommonly so in biopsies of the disease This atypical epithelium if insulted continuously with tobacco or other superimposed injury could lead to oral malignancy (*Pindborg et al* 1966)

The persistent dilatation seen in many moderately advanced and advanced biopsies also merits some comment In oral submucous fibrosis while there is a rise in mast cells in the earlier stages of the tissue reaction the counts are similar to those seen in normal mucosa or even lesser in the more advanced stages (*Sirsat & Pindborg* 1967a 1967b) This would suggest that similar to the situation seen in many delayed reactions the vasodilator amines synthesised by the mast cells do not mediate in the presence of the vascular dilatation and residual oedema seen in the more advanced stages of oral submucous fibrosis Other factors seem to operate in the sustenance of this long term partial vascular response As to what these factors are can be only a matter of speculation at this stage of our knowledge of the etiology of the disease Two among the many that come to mind in the light of the histopathology of the disease are the globulin permeability factor (*Wilhelm* 1965) and the presence of an antigen antibody complex which is known to sustain increased vascular permeability and dilatation and provoke a hyaline degeneration of connective tissue (*Dixon* 1963 1965)

SUMMARY

One hundred and twenty four biopsies from oral submucous fibrosis were studied in detail for the vascular response in relation to histological stage of the disease Normal dilated and constricted blood vessels were seen often in combination in the same section Epithelial changes consequent to impaired nutrition due to narrowed blood vessels have been discussed—as also the probable causes of the persistant vascular dilatation and oedema seen in the absence of inflammation in some advanced biopsies

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STUDIES BY THE NEWT TEST ON CARCINOGENIC PROPERTIES OF FATS

By

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Studies on the effect of heated and otherwise altered fats as carcinogens by the newt test were carried out by one of the present authors (*Arffmann* 1964) who found this short term test sufficiently specific for a screening of chemical compounds given in oil Among the substances tested certain oxidation products especially ethyl linoleate hydroperoxide gave positive results The newt test was still positive for the product obtained by reduction of the hydroperoxide group to an alcohol group

The observations have been followed up in a more systematic screening of oxidation products of fats by the newt test Several parameters were taken into consideration Oleic acid was compared with linoleic linolenic and arachidonic acids which possess two three and four double bonds respectively in the 1 4 position Eleostearic acid with three double bonds in the 1 3 position and the polyene squalene having four 1 5 double bonds were tested Ethyl esters and triglycerids of the acids were compared Excepting eleostearic acid all compounds were tested as native compounds after oxidation to a suitable peroxide content and after reduction of the peroxide groups The results of the studies are reported in the following

MATERIALS AND METHODS

Substances Triolein trilinolein trilinolenin ethyl linoleate and ethyl linolenate were from Unilever Research Laboratories Vlaardingen Holland Some [illegible] of triolein and trilinolein used as basic controls were from The Hormel I[illegible]e Austin Minnesota U S A (All these substances were supplied and kind[illegible] at [illegible] disposal b[illegible] G[illegible]nd tedværket Arhus Denmark) Most [illegible] per cent, trilinolenin only 90 per cent pure Samples of fre[illegible]ed monthly b[illegible] Grind ted ærket.

Ethyl arachidonate w[illegible] a gift from F Hoffmann La R[illegible] Co A G Ba[illegible]e Switzerland.

Ethyl oleate was prepared by A Jart The Dan[illegible]

A sample of squalen (practical Eastman 90 per [illegible] M[illegible]hylcholanthrene (practical grade) was from Sigma Chemical Co[illegible] St Louis Missouri U S A

Th[illegible]s work was sup[illegible] b[illegible] a grant from the *Danish Anti-Cancer League*

TABLE 1

Properties of Substances Tested by the Newt Test

Substance	p.v.	Optical density	Absorption maximum mμ
Ethyl oleate	30	0.3	217
Ethyl oleate hydroperoxide	650	3.5	217
reduced ethyl oleate hydroperoxide	2	3.3	217
Triolein			
Triolein hydroperoxide	1250	3.4	215
reduced triolein hydroperoxide		3.7	215
Ethyl linoleate			
Ethyl linoleate hydroperoxide	1600	13.9	233
reduced ethyl linoleate hydroperoxide		8	233
Trilinolein	100	0.9	233
Trilinolein hydroperoxide	1300	10.8	233
reduced trilinolein hydroperoxide		11.5	233
Trilinolein hydroperoxide	1800	11.9	233
Ethyl linolenate		1.5	234
Ethyl linolenate hydroperoxide	1600	12.8	234
reduced ethyl linolenate hydroperoxide		16.3	234
Trilinolenin	100	3	235
Trilinolenin hydroperoxide	1100	10.9	235
reduced trilinolenin hydroperoxide	40	10.[illegible]	235
Ethyl arachidonate		1	235
Ethyl arachidonate hydroperoxide	800	2.2	235
reduced ethyl arachidonate hydroperoxide		1.8	235
Cholesterol	0	0.1	234
Cholesterol hydroperoxide	900	7.2	234
reduced cholesterol hydroperoxide	0	7.2	234
Squalene	4	6.9	217
Squalene hydroperoxide	875	28.0	212
reduced squalene hydroperoxide	70	27.4	212
Tung oil		147	271

The table gives the peroxide content in μ equiv./g substance (p.v.) and the optical densities for 1 g substance/1 l cyclohexane at absorption maximum.

Most of the peroxides were prepared by simple aeration with atmospheric air. The peroxide content was controlled at suitable intervals and the aeration discontinued when a level about 1000 μ equiv./g had been reached.

Ethyl oleate and triolein were aerated until a peroxide content of about 200 μ equiv./g only. The products were then dissolved in petrol ether and chromatographed on acid alumina. The hydroperoxides were eluted with chloroform.

Cholesterol was oxidized by ultraviolet irradiation in a quartz flask of a glacial acetic acid suspension in an atmosphere of oxygen. After a suitable amount of oxygen had been absorbed, water was added. The precipitate was washed with water and air dried.

The reduction of peroxides was carried out with stannous chloride in ethanol as recommended by *[illegible] & [illegible]* (19[illegible]).

Peroxide determinations were made by the colorimetric thiocyanate method with the exclusion of atmospheric oxygen (*[illegible] & Hartmann* 19[illegible]). The results are expressed as *microequivalents of peroxide per g fat* (peroxide value, p.v.).

Ultraviolet absorption spectra of the substances dissolved in cyclohexane were recorded by means of a Beckman Ratio Recording Spectrophotometer.

The peroxide content (p.v.) and spectral properties (optical densities at absorption maximum for a solution of 1 g/1 l cyclohexane) are summarized in Table 1.

Most of the substances were diluted with arachis oil to a concentration suitable for the injection. The dilutions were easily prepared by simple mixing. *[illegible]*

and its derivatives however are sparingly soluble in arachid oil and since it was desirable to give also these substances in a readily available form in clear solutions only weak concentrations could be prepared One per cent (w/v) solutions were obtained by vigorous mechanical stirring Solutions of methylcholanthrene were prepared in the same way with additional heating

Experimental procedures The technique of the next test has earlier been described in detail (*Arffmann & Christensen* 1961 *Arffmann* 1963 *Arffmann* 1964b) Since maximal positive reactions were found after about two weeks it was found reasonable to simplify the test by amputating all tails on the 15th day Animals dying before were included if three days had elapsed after the injection Of the whole effective number of experimental animals only 8 died before the 7th day

Details concerning the single experiments are given in Tables 2–6 An upper limit for the strength of the test solutions is set by the necrotic lesions produced by high concentrations of the substances (*Arffmann* 1964a) To facilitate comparison the peroxides were tested in dilutions of a calculated p v of 600 The response in relation to the dose was further studied by giving more dilute (p v 200) or when toxicity was low or negligible more concentrated (p v 1100–1800) products The reduced and the native compounds were diluted to match the higher or the highest concentrations of the peroxides and in some instances applied undiluted

Each experiment comprised native and altered compounds within the same group (e g ethyl oleate and derivatives and triolein and derivatives and included negative and positive controls With minor exceptions each compound was injected into 6 animals. As far as possible males and females were equally represented If the quantity of substance and the number of animals were sufficient the experiment was repeated twice In some instances concentrations were altered during this sequence so with the ethyl oleate group because of an unexpectedly high toxicity and with the *squalene group because of similar signs on the 4th day after the injection* of the first animals Low peroxide values of ethyl linolenate hydroperoxide and of trilinolenin hydroperoxide were not tested because of scarcity of substances and animals

RESULTS

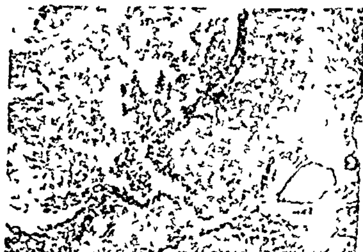
The results are summarized in Tables 2–6 Positive reactions cover + and (+) responses (*Arffmann & Christensen* 1961) while doubtful reactions (±) are not included Effective number of animals results from the total number by eliminating animals died before the 3th day after the injection and animals found unsuited because of severe necrosis cadaverosis or skin inflammation One animal with minimal remnants of the injected oil was also excluded

Local toxic effect of the injected solutions was generally mild or absent Severe necrosis of the tail was found in only 19 animals and these had to be discarded One had been injected with tung oil 10 per cent 11 had received ethyl ester or squalene hydroperoxides and 7 had received the reduced compounds None of the triglyceride derivatives induced such necrotic lesions For the comparison of a strongly toxic lesion with that of a positive and a negative reaction see Figs 1–3

All hydroperoxides of the fatty esters gave positive results by the next test When more dilutions of the peroxides were tested an increasing effect of ethyl oleate ethyl linoleate and ethyl arachidonate with increasing p v was observed The dependence on the peroxide content is also indicated by the results with triglycerides and squalene The responses to the reduced compounds did not however differ significantly from those to the corresponding hydroperoxides The positive results after injection of ethyl linoleate hydroperoxide and the corre


1





sponding reduced compound are quantitatively in keeping with those obtained in earlier experiments (*Arffmann* 1964a)

One of the objects of the investigations was to compare the effect of altered ethyl esters with that of similarly altered triglycerides Derivatives of triolein showed to be distinctly less active than those of ethyl oleate triolein hydroperoxide p v 1250 was the only active concentration A tendency to a lower activity of the triglycerides was also shown by linoleic and linolenic acids A negative result was obtained by trilinolein hydroperoxide p v 600 on the other hand a positive response was shown by native trilinolenin which weakens the significance of the strong reaction to peroxidized and reduced trilinolenin

TABLE 2

Experiments with Oleate Triolein Compounds

Substance	Peroxide content (p v)	Test solution Strength % in ol arach	Calculated p v	Number of experiments	Total number of animals	Results Positive reactions Effective number of animals
Oleum arachidis				2	12	0/12
Ethyl oleate	30	30 8		2	10	0/10
Ethyl oleate	30	92 3		1	6	1/6
Ethyl oleate hydroperoxide	650	15 4	100	2	10	0/10
Ethyl oleate hydroperoxide	650	30 8	200	3	16	7/16
Ethyl oleate hydroperoxide	650	92 3	600	1	6	Necrosis
Reduced ethyl oleate hydroperoxide	2	30 8		2	10	5/10
Reduced ethyl oleate hydroperoxide	2	92 3		1	6	2/2
Triolein		48 0		3	18	0/18
Triolein		100 0		1	6	0/6
Triolein hydroperoxide	1250	16 0	200	3	18	0/18
Triolein hydroperoxide	1250	48 0	600	3	18	1/18
Triolein hydroperoxide	1250	100 0	1250	1	6	2/6
Reduced triolein hydroperoxide	0	48 0	0	3	18	1/18
3 Methylcholanthrene		0 5		3	18	13/18

Figs 1–3

Fig 1 Absence of epithelial reaction after the injection of ethyl oleate 92 3 per cent in arachid oil The oil droplets are represented by empty holes (40×)

Fig 2 Epithelial hyperplasia and infiltrative downgrowth after the injection of ethyl oleate hydroperoxide p v 00 (40×)

Fig 3 Deep necro is of tail after the injectio f th l oleate hydroperoxide p v 600 The ulcerative de ect is covered b t v infiltrative epithelium. The case was discarded. (40×)

TABLE 3

Experiments with Linoleate Trilinolein Compounds

Substance	Peroxide content (p.v.)	Test solution: Strength % in ol. arach.	Test solution: Calculated p.v.	Number of experiments	Total number of animals	Results positive reactions / effective number of animals
Oleum arachidis				2	12	0/12
Ethyl linoleate		37.5		3	17	0/16
Ethyl linoleate hydroperoxide	1600	12.5	200	3	16	1/16
Ethyl linoleate hydroperoxide	1600	37.5	600	3	16	7/16
Reduced ethyl linoleate hydroperoxide	0	37.5	0	3	17	4/15
Trilinolein	100	46.2		3	19	1/16
Trilinolein	100	100.0		1	6	1/6
Trilinolein hydroperoxide	1300	15.4	200	1	12	1/11
Trilinolein hydroperoxide	1300	46.2	600	3	21	0/19
Trilinolein hydroperoxide	1800	100.0	1800	1	6	6/6
Reduced trilinolein hydroperoxide	0	46.2	0	3	18	5/18
3-Methylcholanthrene		0.5		3	18	11/17

TABLE 4

Experiments with Linolenate Trilinolenin Compounds

Substance	Peroxide content (p.v.)	Test solution: Strength % in ol. arach.	Test solution: Calculated p.v.	Number of experiments	Total number of animals	Results positive reactions / effective number of animals
Oleum arachidis				2	10	1/10
Ethyl linolenate		37.5		3	14	1/14
Ethyl linolenate hydroperoxide	1600	37.5	600	3	14	11/12
Reduced ethyl linolenate hydroperoxide	0	37.5	0	2	12	10/11
Trilinolenin	100	54.5		2	8	[illegible]/7
Trilinolenin	100	100.0		1	6	2/5
Trilinolenin hydroperoxide	1100	54.5	600	3	14	10/14
Trilinolenin hydroperoxide	1100	100.0	1100	1	6	6/6
Reduced trilinolenin hydroperoxide	40	54.5		1	6	1/5
Reduced trilinolenin hydroperoxide	40	100.0		1	6	6/6
3-Methylcholanthrene		0.5		2	12	10/11

TABLE 5

Experiments with Ethyl Arachidonate Compounds and Tung Oil

Substance	Peroxide content (1 v)	Test solution		Number of experiments	Total number of animals	Results positive reactions effective number of animals
		Strength % in ol arach	Calculated pv			
Oleum arachidis				2	8	0/5
Ethyl arachidonate		75.0		2	12	1/8
Ethyl arachidonate hydroperoxide	800	25.0	200	2	12	2/8
Ethyl arachidonate hydroperoxide	800	75.0	600	2	12	5/10
Reduced ethyl arachidonate hydroperoxide	0	75.0	0	2	12	5/7
3-Methylcholanthrene		0.5		2	12	8/12
Tung oil		10.0		2	12	8/11
Tung oil		25.0		1	6	5/5

A comparison of the four ethyl ester hydroperoxides indicates that oleate and linolenate have the strongest effect on the animals while linoleate and arachidonate exhibited lower activity. Turning to the reduced compounds we find the ethyl linolenate more active than the other three compounds. In contrast the results with the triglyceride hydroperoxides and the corresponding reduced compounds indicate an increasing effect with increasing number of double bonds of the component acids.

Results with altered squalene are shown in Table 6 and have been mentioned above. Responses were strongly positive. Cholesterol hydroperoxide and the reduced compound had no effect on the newt skin probably on account of the weak concentrations applied.

Tung oil was tested in three experiments (Table 5). Because of local toxicity the primarily applied 25 per cent dilution was replaced in later experiments by the less toxic 10 per cent dilution in arachid oil. In both instances the response was strongly positive.

In general injections with arachid oil and with cobalt red fats had no effect and so served their purpose as negative controls. A single positive reaction does not influence a negative judgment unless it can be reproduced (*Arffmann* 1964b). On the basis of this principle native trilinolein may be estimated as doubtfully positive while only trilinolenin has shown a weakly to moderately positive activity. The preparations of native trilinolein and trilinolenin showed however a rather high peroxide content which may well explain the positive results. Injections with methylcholanthrene in all instances had a strongly positive effect and so confirmed the reactivity of the animals.

TABLE 6

Experiments with Cholesterol Squalene Compounds

Substance	Peroxide content (p.v.)	Test solution		Number of experiments	Total number of animals	Results Positive reactions/ Effective number of animals
		Strength % in ol arach	Calculated p.v.			
Oleum arachidis				3	18	0/18
Cholesterol	0	1 0		3	18	0/18
Cholesterol hydroperoxide	900	1 0	9	3	18	1/18
Reduced cholesterol hydroperoxide	0	1 0	0	3	18	1/18
Squalene	4	50 0		2	12	1/11
Squalene	4	68 6		1	6	0/5
Squalene hydroperoxide	875	50 0	437 5	2	12	6/10
Squalene hydroperoxide	875	68 6	600	1	6	6/6
Reduced squalene hydroperoxide	70	50 0		2	12	9/12
Reduced squalene hydroperoxide	70	68 6		1	6	2/5
3-Methylcholanthrene		0 5		3	18	13/17

DISCUSSION

An inspection of the figures of Tables 2–6 shows quite conclusively that evidence of carcinogenic properties is shown by three groups of substances 1 The hydroperoxides 2 The corresponding reduced compounds 3 Tung oil

The rather close identity of the responses produced by the hydroperoxides and by equal amounts of the corresponding reduced compounds shows that the hydroperoxide group is not a prerequisite for the carcinogen like effect The hydroperoxide group is supposed to be converted into an alcohol group through the reduction with stannous chloride A reduction to an alcohol group *in vivo* could be a plausible explanation for the similarity between the responses of the hydroperoxides and the reduced compounds If so the active substances would be fatty acids having a hydroxyl group in *a* position to a double bond

In this connection it should be mentioned that *van Duuren et al* (1963 1965) found certain epoxides and peroxides carcinogenic to the skin of mice They found activity of 1 hydroperoxycyclohex-3 ene and mention that carcinogenicity has earlier been reported for two further *a β* unsaturated hydroperoxides *Kotin & Falk* in 1963 reported preliminary data of tumorigenic response in mice after exposure to selected organic peroxides the strongest effect being elicited by m and p-tert butyl isopropylbenzene hydroperoxide

On the other hand a strong effect was also shown in our experiments by tung oil which does not possess hydroxyl groups The characteristic acid of this oil is elaeostearic acid with three conjugated double bonds The isomeric linolenic acid has three methylene interrupted double bonds and is inactive Conjugation however takes place during autoxidation of the fatty acids with more double bonds The optical density at about 233 mμ gives an expression of the amount of conjugation (Table 1) The conjugation remains after reduction and about the same maxima of absorption and optical densities were found in the hydroperoxides and the corresponding reduced compounds All conjugated fatty esters exhibited carcinogen like activity

An effect was however also shown by the hydroperoxides of oleic esters and squalene and the corresponding reduced compounds Conjugated double bonds are not formed during autoxidation of oleic acid or squalene and it can be concluded from the spectral properties that they did not contain significant amounts of conjugated dienes as by products or impurities either On the other hand the hydroperoxides and the reduced compounds of oleic acid gave much higher optical densities at 212–214 mμ than the corresponding non oxidized compounds Spectrophotometric studies on the oxidation of oleic acid and its esters have been carried out by *Holman et al* (1945) Their spectra also show a higher extinction at about 200 mμ for oxidized ethyl oleate than for the fresh compound but they do not discuss the reason for the increased absorption at this wavelength

A second kind of isomerization besides conjugation which also takes place during the autoxidation process is formation of trans isomers from the native cis compounds A cis trans isomerization takes place during autoxidation of all unsaturated fatty acids and also tung oil contains double bonds in trans configuration Although an estimate of the extent of the trans configuration in the products was not made there can be no doubt that a high degree has been present in all the active products

The most thoroughly studied class of chemical carcinogens are the polybenzene hydrocarbons Although this group of substances is chemically very distant from the altered fatty acids an important similarity between the two groups of lipid soluble substances exists both possess π electrons The π-electrons are probably in both cases responsible for the carcinogenic properties but also other requirements must be fulfilled A special electronic configuration seems to be required for carcinogenic activity of the hydrocarbons but a complete agreement with chemical predictment has so far not been obtained The possible importance of a hydroxyl group in α position to a double bond of conjugation of the double bonds and of trans configuration has been discussed above The final identification of the configuration or configurations responsible for the activity can only be made by further screening of more substances

The presence of a hydroxyl group generally cancels the activity of the hydrocarbons (*Falk et al* 1962) but the negative effect may be due to a more rapid elimination from the organism of the hydroxylated compounds On the contrary all our active compounds with the exception of tung oil contain a hydroxyl or a hydroperoxide group Metabolic relationships may however also play a role for the activity of altered fats The tendency to a smaller activity of the triglycerides in comparison with the corresponding ethyl esters may be due to a more rapid attack by lipase as the first metabolic step but of course also to different physico chemical properties

Due to the sparing solubility in arachid oil the conversion products of cholesterol were tested in only weak concentrations and no activity was found Conversion products of cholesterol have been rather extensively studied for carcinogenic effects (*Fieser* 1954) The point of departure for the studies has generally been a similarity with chemical carcinogens *with respect to ring structure Our results indicate that* the clue for the nature of carcinogenicity of lipids must rather be sought in the configuration around the double bonds in general and those of the fatty acids in particular

Although the indication of carcinogenic properties of altered fatty acids has been obtained only by the newt test it would seem to be of considerable interest that such minor configurative changes or substitutions *in essential or ubiquitous substances of organisms* as the essential fatty acids may result in dangerous qualities The literature contains a variety of reports on toxic properties and suspected carcinogenicity of altered fats but studies of the effects of specific alterations of the fatty acids are more scarce We found no great acute toxicity of parenteral injection of a variety of lipoperoxides in rats and chicks (*Glavind Sondergaard & Dam* 1961) Rather high amounts of lipoperoxides are present in rat skin surface lipids (*Glavind & Christensen*) The effects of isomerized fatty acids have been studied especially in nutrition al experiments in relation to essential fatty acids (*Aaes Jorgensen* 1966) The identification and separation of the isomerized or otherwise altered products of unsaturated fatty acids is made possible by recent methods The possibility of carcinogenic effect might give new impetus to a study of the biology of altered fats based on chemically well identified substances and studies on these compounds may on the other hand throw new light on the mechanisms of carcinogenesis

SUMMARY

Carcinogenic properties of derivatives of fatty acids and certain other lipids were studied by the newt test The ethyl esters of oleic linoleic linolenic and arachidonic acids the hydroperoxides of the esters and the compounds obtained by reduction of the hydroperoxides were tested All the hydroperoxides and the reduced compounds were active

Slightly smaller activities were shown by the hydroperoxides of triolein trilinolein and trilinolenin and the corresponding reduced compounds High activities were shown by squalene hydroperoxide and its reduced derivative and by native tung oil

The results are commented and the possible importance of a hydroxyl group in α position to a double bond of conjugation of double bonds and of trans configuration for carcinogenic properties of lipids is discussed

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LACTIC ACID DEHYDROGENASE ISOENZYMES IN HUMAN MELANOMAS

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Received 7.ix.66

By combination of histochemical and electrophoretic techniques the demonstration of isoenzymes with substrate specificity is possible. Measurement of isoenzyme activities may yield more information than simple measurement of total activities, as the individual isoenzymes may be altered in opposite directions leaving the total activity nearly unchanged.

Under pathologic conditions the cells of damaged organs may release some of their intracellular enzymes into the bloodstream. An increased amount of lactic acid dehydrogenase (LDH) in serum of patients with advanced malignant tumours were found by *Hill & Levi* (1954). Changes in serum LDH isoenzymes are seen in myocardial infarction with cell necrosis as demonstrated by *Wieme* (1959), *Laursen* (1962), and *van der Helm* (1962). In malignant effusions changes have been described by *Richterich et al.* (1962), and alterations in the isoenzyme pattern of brain tumour homogenates have been shown by *Gerhardt et al.* (1963). Patterns of LDH isoenzymes in tumours of the stomach, colon, mammae, kidney, lung, and prostate in 20 patients have been demonstrated by *Nissen & Bohn* (1965).

A number of different isoenzymes in amelanotic and pigmented melanomas of hamsters and one human pigmented melanoma have been investigated by *Pandov & Dilov* (1966). The average per cent of the LDH isoenzyme pattern of 7 human melanomas have been presented by *Bohn & Rothenborg* (1966).

The present investigation is an attempt to differentiate and compare the LDH isoenzyme pattern of pigmented human melanomas to their histology and histochemistry in relation to normal human skin with regard to lactic acid dehydrogenase.

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MATERIAL AND METHODS

Twelve pigmented primary malignant melanomas as defined by *Allen & Spitz* (1953) 6 cases of lymphnode metastasis and 12 skin specimens from different persons were investigated as to their contents of LDH isoenzymes All primary tumours and the skin specimens have been painted with alcoholic iodine before surgical removal Immediately after extirpation the tissues were carefully prepared for homogenization by an electric glass homogenizer being cooled by ice water Subcutaneous fat was removed from the normal skin by scraping lightly with a knife in order to obtain comparable specimens After centrifugation (10 000 r p m) the clear supernatant was used for agar gel electrophoresis as described by *Wieme et al* (1962) Samples were placed in Difco Noble Agar buffered to pH 8 6 by diem l natrium buffer Two by two the slides were immersed in a bath of light petroleum for cooling purposes and electrophoresis was performed at 140 volt for 30 minutes The slides were incubated at 37 together with frozen sections from the same tissues and stained by nitroblue tetrazolium DPN techniques using rubber frames as described by *Bohn & Rothenborg* (1966) *The histology of the specimens was further* controlled in haematoxylin eosin stained frozen and paraffin sections The staining of the electrophoresis was fixed in acetic acid ethanol at room temperature for 120 minutes and dried Scanning was performed at a Densicord (Photovolt) at 530 nm The coloured bands were numbered from the anode and their enzyme activity expressed as percentage of total activity All samples were run as duplicates

TABLE 1

LDH Isoenzyme Pattern in 12 Human Malignant Melanomas Enzyme Activity Is Expressed as per cent of the Total Activity

Bands	I	II	III	IV	V
Slide No		*Anode*		*Cathode*	
16 037/6	7	22	29	24	18
489/66	7	21	28	26	18
684/66	8	22	26	24	20
1 016/66	3	17	33	29	18
1 332/66	3	8	28	33	28
3 170/66	0	3	22	36	39
4 875/66	2	17	25	29	27
4 891/66	2	15	26	28	29
6 071/66	8	9	32	29	22
6 241/66	3	14	30	29	24
6 679/66	24	24	24	21	7
7 772/66	0	9	33	35	23
Average per cent	[illegible]	15 1	[illegible] 0	[illegible] 6	[illegible]

RESULTS

Table 1 presents the results of the LDH isoenzyme activity of 12 human pigmented melanomas The enzyme activity is expressed as per cent of total activity In all cases but two 5 bands of LDH activity were present The main activity was found among the cathode bands in all tumours In 7 cases the greatest amount of activity was found in band 3 while 3 tumours appeared to show the greatest amount of isoenzyme in band 4 and 2 in band 5

The relative activity of LDH isoenzyme distribution in lymphnode metastasis from malignant melanomas is given in Table 2 It is seen

that the main portion of the isoenzyme pattern is found among the cathodal bands. In all cases 2 anodal and 3 cathodal bands are present.

Table 3 demonstrates the LDH isoenzyme pattern in normal human skin. It is seen that also for this specimen the main activity is found among the cathodal bands. It appears that none of the specimens investigated contained more than one anodal band.

TABLE 2

LDH Isoenzyme Pattern in 6 Lymphnode Metastases from 6 Human Malignant Melanomas. Enzyme Activity Is Expressed as per cent of Total Activity

Bands	I	II	III	IV	V
Slide No	*Anode*			*Cathode*	
15 157/65	14	22	[illegible]	21	18
79/66	5	7	28	33	27
4 001/66	13	22	31	24	10
5 435/66	20	[illegible]	26	18	11
5 884/66	7	21	31	25	16
6 071/66	5	10	34	1	20
Average *per cent*	*10 7*	*17 8*	*29 2*	*25.3*	*17 0*

TABLE 3

LDH Isoenzyme Pattern in 12 Human Skin Specimens. Enzyme Activity Is Expressed as per cent of Total Activity

Bands	I	II	III	IV	V
Slide No	*Anode*			*Cathode*	
4 875/66	0	8	[illegible]	26	44
4 881/66	0	4	27	32	37
4 891/66	0	0	36	26	38
4 903/66	0	2	28	32	38
5 062/66	0	21	36	43	0
5 066/66	0	15	47	36	2
5 840/66	0	2	45	53	0
6 071/66	0	0	23	32	45
6 146/66	0	0	58	42	0
6 38/66	0	0	31	47	22
6 241/66	0	0	32	32	36
6 541/66	0	0	19	43	38
Average *per cent*	*0 0*	*4 3*	*33 7*	*37 0*	*25 0*

The histologic control showed that all tumours investigated were malignant melanomas. The skin specimens did not present pathologic changes. The lymphnode metastases were all from patients with a primary histologically verified malignant melanoma and the microscopic pictures were of typical structure. The highest LDH activity was found in tumour cells compared with other types of cells present in the sections stained by histochemical methods.

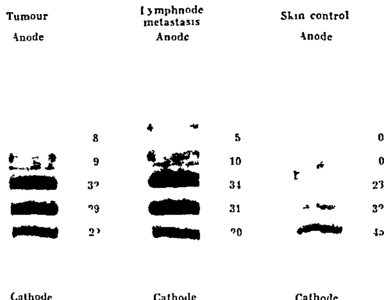


Fig 1

The LDH pattern in primary tumour Lymphnode met stasis and sl in control from the same patient (6 071/66)

Enzyme activity is expressed as per cent of total activity on level with the bands demonstrated

DISCUSSION

Different types of LDH have been found by *Cahn et al* (1962) and by *Fine et al* (1963) The LDH isoenzyme pattern during fetal development has been described by *Philip & Vesell* (1962) in chick liver and muscle The changes were characterized by a progressive increase of the cathodal bands relative to anodal bands *Jensen & Thorling* (1965) suggested that changes in oxygen supply may alter the LDH pattern as they found that the isoenzymes in anaemic and cobolt treated rabbits were found to change gradually from prominence of the anodal to a *prominence of the cathodal isoenzymes compared to control animals*

In malignant tissue *Gerhardt et al* (1963) described a more cathodic pattern of LDH isoenzymes from malignant brain tumour homogenates compared to normal brain tissue Similar results are presented by *Nissen & Bohn* (1965) from investigation of surgical biopsy material from tumours of the stomach colon r a lines lung and prostate compared with controls

In the present study the main activity in the LDH pattern of malignant melanomas (Table 1) and normal skin (Table 2) showed a dominance of the cathodal isoenzymes From Table 1 it appears that all melanomas except 2 showed two anodal bands The average of these is essentially higher than the average of the skin controls where only 1 anodal band was demonstrated All skin covered specimens were painted with alco-

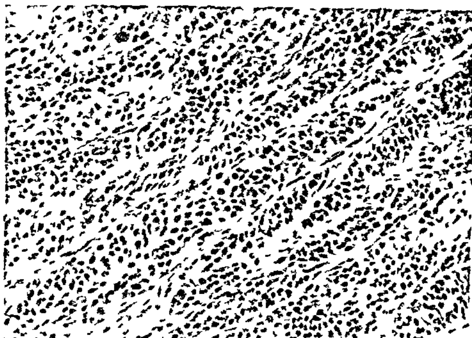


Fig 2

Fig 2 demonstrates the amount of fibrous tissue and inflammatory cells in melanotic tumour tissue used for homogenization (haematoxylin-eosin × 300)

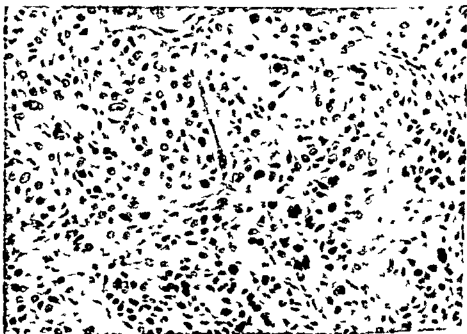


Fig 3

Fig 3 shows only small amounts of other cells in tumour tissue used for homogenization (Haematoxylin eosin × 3?0

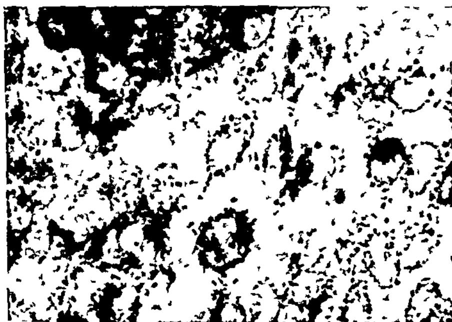


Fig 4

A large amount of lactic acid dehydrogenase is demonstrated as colour precipitates in the cytoplasm of the tumour cells (Nitroblue tetrazolium × 3200)

holic iodine but this comprised the surface of the tumours so that it does not explain the differences found. An increase of the anodal bands relative to the cathodal bands of the melanomas may therefore indicate an alteration of the LDH isoenzymes in proliferating tissues of the skin. This is supported by the investigation of *Bohn & Rothenborg* (1966) who in 8 basal cell carcinomas and 31 plantar warts also found a change towards the anode in the average LDH isoenzyme pattern compared with normal skin.

The LDH pattern in lymphnode metastases from malignant melanomas are given in Table 2. In all cases 5 bands of activity were present. Only 4 bands of activity, however, have been described by *Landov & Dikov* (1966) in 2 cases of lymphnode metastases from melanomas. The LDH pattern of the lymphnode metastases investigated is very much alike that of the primary malignant melanoma. Fig. 1 demonstrates the LDH pattern in a primary tumour and lymphnode metastasis compared to skin control from the same patient. It appears that the total activity in skin judged from the staining of the bands is much less than in the tumour tissues. It is further noted that the values for enzyme activity in the melanoma and the metastasis demonstrated on Fig. 1 are very close. This may suggest a possible way of identifying metastasis from an unknown primary tumour among which the melanomas may give rise to very anaplastic and polymorph metastases. As

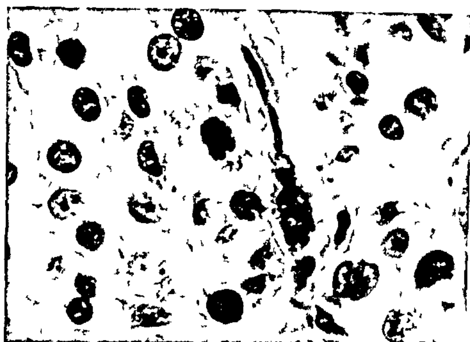


Fig. 5

The individual variation in size, shape and nuclear detail seen in some melanomas.
(Haematoxylin eosin × 3 200)

it appears from tables given the variation in figures is rather wide. Therefore the LDH isoenzyme pattern of a great number of tumours and metastases from the cathodal increasing group should be investigated and results not only presented as average values but with full attention to individual variations.

Finally, the histology of the tumours was compared with the patterns of LDH. No correlation was found with regard to the histological structures though the variation among the different types of melanomas investigated was large as demonstrated on Figs. 2 and 3. It is seen that the histologic control of the tumours revealed that to some extent also other tissues were present but to a varying degree. Thus fibrous tissue containing a number of inflammatory cells as necrotic neoplastic areas were found although grossly the tissue investigated appeared to consist of tumour tissue only. These findings however did not show any correlation to the isoenzyme pattern found, which was dominated by the neoplastic lesion. This is in accordance with the histochemical studies of the LDH in sections from the same tissue. A large amount of enzyme was found in the tumour cells as demonstrated in Fig. 4. Although other cells than the cancer cells contained LDH the precipitate of blue coloured formazan was much more pronounced in the neoplastic cells, which great variety in size and nuclear detail is demonstrated in Fig. 5.

SUMMARY

By means of agar gel electrophoresis combined with histochemistry using artificial electron transport systems as staining procedures (Nitro-BT DPN) the isoenzyme pattern of lactic acid dehydrogenase was examined in homogenates from 12 human pigmented melanomas 6 cases of lymphnode metastasis and 12 specimens of normal human skin as controls The isoenzyme activity was expressed as per cent of total activity and the electrophoretic bands numbered from the anode All tissues investigated had been controlled by light microscopy

The melanomas were found to have an increase of anodal bands relative to cathodal bands compared with normal skin which may indicate alterations of the LDH isoenzymes in proliferating tissues of the skin This is in contrast to several tumours of other origin where a relative increase of the cathodal fractions are found The lymphnode metastasis showed an isoenzyme distribution similar to that of malignant melanomas This may suggest possibilities of identifying metastasis of an unknown primary tumour among which the melanoma may give rise to very anaplastic and polymorph metastatic lesions

The differences in histology of the tumours and lymphnode metastasis were not found to show correlations to the LDH pattern of the specimens investigated The histochemical stained frozen sections gave evidence that the greatest amount of LDH was present in tumour cells compared to other cells found in the same section

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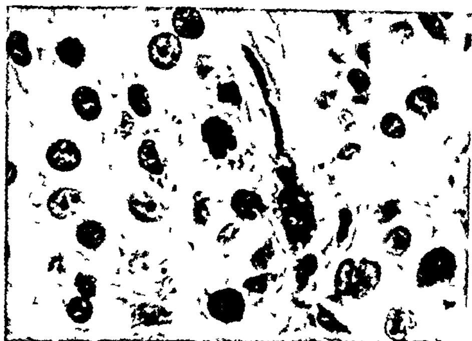


Fig 5

The individual variation in size shape and nuclear detail seen in some melanomas (Haematoxylin eosin × 3 200)

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Finally the histology of the tumours was compared with the patterns of LDH No correlation was found with regard to the histological structures though the variation among the different types of melanomas investigated was large as demonstrated on Figs 2 and 3 It is seen that the histologic control of the tumours revealed that to some extent also other tissues were present but to a varying degree Thus fibrous tissue containing a number of inflammatory cells as necrotic neoplastic areas were found although grossly the tissue investigated appeared to consist of tumour tissue only These findings however did not show any correlation to the isoenzyme pattern found which was dominated by the neoplastic lesion This is in accordance with the histochemical studies of the LDH in sections from the same tissue A large amount of enzyme was found in the tumour cells as demonstrated in Fig 4 Although other cells than the cancer cells contained LDH the precipitate of blue coloured formazan was much more pronounced in the neoplastic cells which great variety in size and nuclear detail is demonstrated in Fig 5

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THE FINE STRUCTURE OF THE RENAL GLOMERULUS IN ACUTE ANURIA

By

T. Steen Olsen and H. Skjoldborg

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In acute renal failure the clearance values for the usual filtration substances are very low, but give only minimum values for the size of the filtration, because the possibility of a back diffusion of the ultrafiltrate cannot be eliminated. Nevertheless, it is the general assumption that glomerular filtration during the anuric phase is likely to be strongly diminished (*Brun* 1954).

Light microscopic studies have not shown any pathological lesion of the glomeruli in acute anuria (*Brun & Munck* 1957). Electron microscopic investigations made in only a few patients showed glomerular components to be normal (*Dalgaard* 1960, *Laguens & Miatello* 1964). Regnier (1959) in one case found endothelial hypercellularity. In order to extend and supplement these earlier investigations a study of 12 renal biopsies from 10 patients with acute renal failure was undertaken. The findings were compared with biopsies from 5 patients with normal kidney function.

MATERIAL AND METHODS

The patients. Detailed d[illegible]s[illegible]r[illegible]pti[illegible]n of the clinical and laborat[illegible]ry data of th[illegible] patients is published elsewhere (*St[illegible]en Olsen* 1967). The patients wer[illegible] [illegible] [illegible] and 69 years old. The acute renal failure appeared a[illegible] [illegible] of surgical o[illegible]bstetr[illegible]cal operations ([illegible] pat[illegible]ent[illegible]), some of them [illegible]ted b[illegible] s[illegible]psis, or it f[illegible]llowed poisoning wit[illegible] [illegible]ulf[illegible]namides, barbiturate or [illegible] tranq[illegible] [illegible] (Truxal) (3 patients). In 7 of the p[illegible]tient[illegible] clinicall[illegible] manifest [illegible] wa[illegible] [illegible]bserved.

The biopsies were perf[illegible]rmed between the 2nd [illegible] the [illegible] [illegible] [illegible]. Two of the patients were b[illegible]op[illegible] [illegible]. Two b[illegible]opsies were [illegible] [illegible] [illegible]. F[illegible]ght patients h[illegible]d b[illegible]en tr[illegible]d with hem[illegible] dial[illegible] [illegible]. All p[illegible]t[illegible]nt[illegible] had received infusion of [illegible] c[illegible]nt gluco[illegible]e solut[illegible] [illegible]. Three of them ha[illegible] [illegible]lso [illegible] treated with Rhe[illegible] [illegible]ex. In [illegible] [illegible] resulted in light m[illegible] [illegible] [illegible]smot[illegible]c change[illegible] [illegible].

The r[illegible]nal biops[illegible] w[illegible]e [illegible]rmed under [illegible] [illegible] [illegible] [illegible] [illegible]n[illegible]edle through a small [illegible] on in th[illegible] right lumbar [illegible].

This work wa[illegible] [illegible]ded b[illegible] [illegible] *Dan[illegible]sh Scien[illegible]e Found[illegible]tion* [illegible] *Michaelsen Foundation*.

We are indebt[illegible] to civil [illegible]neer Mr. *A. Birch And[illegible]sen* f[illegible] [illegible]uable advice and to laboratory te[illegible]nician Mr. [illegible] *Ped[illegible]r[illegible]en* for the ph[illegible]tograp[illegible] [illegible]productions.

The controls These 5 patients were between 12 and 33 years of age In 4 of the patients the biopsies were performed during laparatomy made for other purpose (appendicitis cholelithiasis hysterectomy) with same needle as that used for the anuric patients The fifth control was a donor for renal transplantation and here a slice was cut from the left renal cortex just after death All control patients had normal renal function and no abnormal constituents in the urine

Light microscopy A part of the tissue cylinder was fixed in *Helly's* fluid mixed just before use from the stock solution and the formaldehyde solution Following fixation which lasted exactly one hour the tissue was rinsed in distilled water for 12-18 hours Dehydration in alcohol treatment with xylol embedding in paraffin. Serial sections of a thickness of about 2 3 micron were cut using a rotating microtome Routine stainings were Hematoxylin eosin Sirius red—iron hematoxylin periodic acid – Schiff – hematoxylin phosphotungstic acid – hematoxylin for fibrin methyl violet congo red and thioflavine (fluorescence) for amyloid

Electron microscopy Ten to twenty small pieces were cautiously cut from each end of the tissue cylinder *with a razor blade and immediately fixed by immersion in* OsO_4 *(4 patients) or in glutaraldehyde (the remaining) Four of the controls were* fixed in OsO_4 one in glutaraldehyde

Osmic acid fixation was performed for one hour in OsO_4 1 0 per cent in *Michaelis* buffer (pH 7 3) Washing in the buffer

Glutaraldehyde fixation was in 1 0 per cent glutaraldehyde at room temperature for one hour Rinsing several times in 0 1 molar cacodylate buffer (pH 7 4) One hour postfixation in 1 per cent OsO_4 dissolved in the same buffer Washing with cacodylate buffer containing sucrose 0 2 molar Both types of fixation were followed by dehydration in acetone embedding in *Vestopal W* The specimens were sectioned with a *LKB Ultrotome* stained at the copper grid with uranyl acetate for ½ hour Microscope with a *Philips* EM 200

RESULTS

Light Microscopy

A detailed description of the light microscopic and electron microscopic lesions of the tubules in these biopsies has been published by *Steen Olsen* (1967) All biopsies presented light microscopic lesions similar to those described earlier by *Brun* (1954) and *Brun & Munck* (1957) All biopsies contained light microscopic normal glomeruli in the patients as well as in the controls

Electron Microscopy

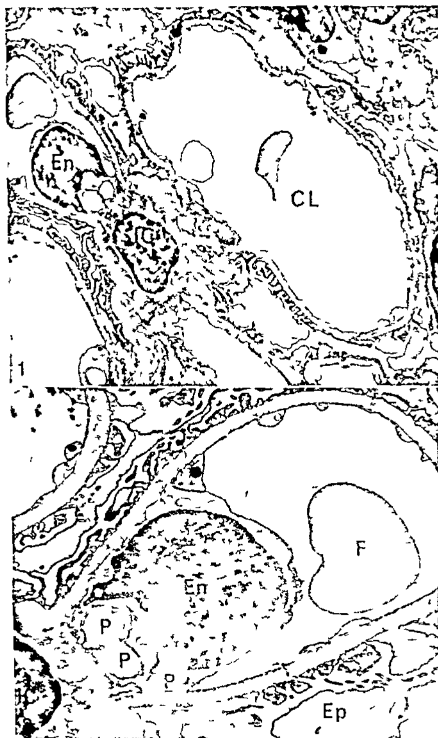
No significant abnormalities of the basic structure of the glomeruli were observed (Figs 1 4) The capsular epithelium was flat The cellular components of the capillary tuft were in most biopsies of normal size and positions the capillary lumina were patent and con

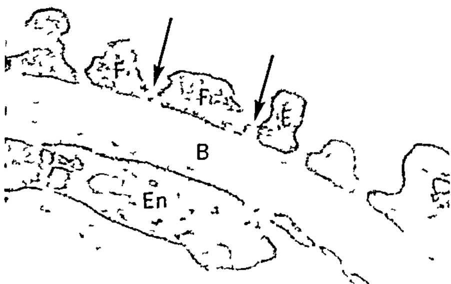
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Figs 1 2

Fig 1 Glomerular capillaries of normal structure En endothelial cell nucleus IC intercapillary cell nucleus CL capillary lumen [illegible] 5th day of anuria Glutaraldehyde fixation E900 ×

Fig Same case part of the endothelial cell capillary lumen at the left in Fig 1 Normal structure of all elements En endothelial cell nucleus [illegible] from intercapillary cell IC intercapillary cell nuclei Ep epithelial cell cytoplasm R red blood corpuscle Note the patent capillary lumen the wide urinary space and foot processes with no fusion Glutaraldehyde fixation 15000 ×


En
CL
1
En
F
P
P
P
Ep



4

tained plasma and some red blood corpuscles Thrombi or fibrin deposits were not present The epithelial foot processes were not fused the intervening filtration slits could be demonstrated The basement membrane was of normal thickness and strucure

Some epithelial cells from cases of anuria as well as from controls contained 1–4 micron large inclusions in the cytoplasm consisting of vacuoles containing smaller homogenous rather electron dense globules and in some sections small amounts of granular material (Figs 5 and 6) The mesangial cells (intercapillary cells) were of normal size and structure They were situated in a network of branches of basement membrane like material and often plump processes of rather clear cytoplasm could be seen projecting into the capillary lumina covered or uncovered by endothelial cytoplasm (Fig 2)

The following deviations from the normal structure were noted

In two biopsies (Nos 1 and 2 both from the second day of anuria) a swelling of the epithelial cytoplasm was observed which filled up the capsular space changing this to a narrow slit (Fig 7) Normally some epithelial cytoplasmic processes are present in which the structure is uniform and of a medium electron density without organelles or ribosomes These processes were the cell constituents which seemed to be expanded in the two cases None of the other ten biopsies showed this phenomenon It could not be demonstrated by light microscopy of Helly fixed tissue One biopsy (No 3 3 days anuria) showed hyperplasia of the intercapillary cells and of the endothelial cytoplasm with narrowing of the capillary lumina (Fig 8) The number of intercapillary cell nuclei seemed to be increased No light microscopic changes were noted in this case no mitoses of the intercapillary cells were seen In all other biopsies the capillary lumina were patent and of normal dimensions They sometimes contained red blood corpuscles in an amount which was equal to that of normal biopsy tissue

In all biopsies from patients with anuria the abnormal presence of *cytosomes* in the epithelial cytoplasm and more seldom in the endothelial and intercapillary cells was noted (Figs 5 6 and 8) They were of the same type as those normally present in many cells i e in the renal tubules of normal rats (*Ericsson* 1964) They could readily be distinguished from the normally occurring polyglobular vacuoles in

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Figs 3–4

Fig 3 Same case Normal con tituents of epitr l al cytoplasm p eserved foot processes normal capillar es Ep nucleu o epithel al cell In normal occurring globular inclusion w th dense bodies mv multivesi ular bo'y Go Golgi zone En nucleus of endothelial cell. Glutaraldehyde fixation 15000 ×

Fig 4 Biopsy N 9 eight day of anuria Normal capillar wall w th all c nst tuents preserved F epithelial foot processes B cap llary ba ement membrane En end helial cell cytoplasm Arrow points toward me nb a s in filtration sl t *Glutaraldehyde fixation 85000* ×


Cy



Fig 7

Biopsy No 2 second day of anuria Epithelial cytoplasm of uniform finely granular structure without organelles (marked by asterisks) occurs normally in human glomeruli but in this ca e it is swelled filling out the capsular space Bo basement membrane of Bowman s capsule CL capillary lumina Ep nucleus of epithelial cell Glutaraldehyde fixation 8000 ×

the cytoplasm of glomerular epithelial cells The cytosomes presented as single membrane bounded vacuolar structures of about ¼ to 2 micron The space inside the vacuoles could be electron optically empty or finely granular with a few electron dense inclu ons of varia ble shape granular fibrillar homogenous and concentric myelin like Identifiable cell organelles or remnants of such were not ob erved In some cases the electron dense matter totally filled the vacuoles

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Figs 5–6

Fig 5 Cytosome (C) i ithel al cytoplasm A p nts t a m nted membranou m te In the right u ad ant a l g bular inclusion a t r ormally occu n the human gl m To the left a p r of an hel al nucle sy No 7 oth da t nar a G utaral dehyde fi ion 25 ×

Fig 6 Same case F g ytosome contain ng myelin li ous material in epithel toal n Glu aral lehyd fixation 43 3



Fig 8

Biopsy No 3 third day of anuria Capillary lumen occluded by swelling of endothelium and intercapillary cells This phenomenon was observed only in this case Compare with Fig 2 En nucleus of endothelial cell IC nucleus of intercapillary cell Cy cytosomes Us urinary space Glutaraldehyde 7000 ×

All nuclei were preserved No necrotic cells were observed Sometimes the urinary space especially near the tubular pole contained fragments of tubular epithelial cytoplasm evidently an artefact produced by the handling of the tissue before fixation

DISCUSSION

The glomeruli in this ultrastructural study of anuria were compared with those in 5 biopsies from patients without renal abnormalities In addition they were compared with the observations of the normal human glomerulus by *Bergstrand & Bucht* (1958) and with an exten

sive recent study of the normal fine structure of the human glomerulus by *Jorgensen* (1966)

Five patients with anuria described by *Dalgaard* (1960) did not show any glomerular lesions The present study showed hyperplasia of epithelial cell cytoplasm in two and hyperplasia and proliferation of intercapillary (mensangial) cells with narrowing of the capillary lumina in one out of 12 biopsies One patient described by *Regnier* (1959) had proliferation of the endothelial cells (intercapillary cells?) Perhaps these changes are to be regarded as cellular modifications connected with the sudden decrease of filtration Since the other biopsies from patients with anuria did not show these changes they cannot be essential for production of the anuric state

All biopsies from patients with anuria showed the presence of cytosomes in the epithelial cytoplasm and more rarely in the endothelial and intercapillary cell cytoplasm These were not observed in the 5 control patients Such cytosomes are presumed to be lysosomes and to contain hydrolytic enzymes Their presence in cells were they do not ordinary occur could be interpreted as a sign of augmented destruction of normal cellular components This non specific type of damage was the only one which was seen in all the biopsies None of the observed abnormalities in glomeruli seem to be of primary importance in the production of the lowered filtration which is presumed to occur in anuria

SUMMARY

Twelve biopsies from ten patients with acute anuria were studied by electron microscopy No severe lesions were observed Two biopsies showed swelling of epithelial cytoplasm In one case capillary lumina were occluded by swelling of endothelium and intercapillary cells These three biopsies were all from early phases of anuria In all other biopsies the glomeruli showed only slight non specific damage of the cells The fine structure of the glomeruli in acute anuria cannot explain the lowered filtration rate which is presumed to occur in acute anuria

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ELECTRON MICROSCOPIC AND CYTOCHEMICAL STUDIES OF ACID PHOSPHATASE AND ARYL SULFATASE DURING AUTOLYSIS

By

JAN L E ERICSSON PETER BIBERFELD & ROLF SELJELID

Received 30 ix 66

Autolysis implies autodigestion of cells and is generally considered to be operative in the deterioration of cell structure and function in the dead organisms An increasing body of evidence (9 12 15 18 27 32–34 36 37) indicates that different lytic enzymes—cathepsins and proteases amino peptidases sulfatases acid phosphatase and others—which are included in the group of lysosomal enzymes (8 9) are located in specific cytoplasmic organelles often termed "cytosomes and "lysosomes (18) It appears that during *in vivo* conditions all the lysosomal enzymes are enclosed in the cytosomes and do not have access to substrates in the surrounding cytoplasm (9) Although it has been suggested by some (6 8 10 26 40) that release of lysosomal enzymes from cytosomes may initiate the degenerative cellular alterations during autolysis and necrosis unanimous agreement on this point has not been reached (37)

The introduction of methods for the fine structural demonstration of acid phosphatase appeared to aid in elucidating the role of lysosomal enzymes during different physiologic and pathologic alterations of cell function However the use of acid phosphatase as a lysosomal marker is hampered to some extent by the fact that it is presently not known whether all the lysosomal enzymes are present in all the single membrane limited bodies (cytosomes lysosomes etc) known to contain acid phosphatase (9)

The possibility (22) of demonstrating a second lysosomal enzyme splitting *p*-nitrocatechol sulfate (an aryl sulfatase with [illegible] intracellular localization appears to be of great [illegible] reactions of lysosomal enzymes as a group Thus [illegible] demon

This stud[illegible] aided by [illegible] grant from *the Swedish Medical Research Council* (project number 1 x 1096 01) The technical assistance of *Miss* [illegible] is gratefully acknowledged

stration of two lysosomal enzymes in the same organelle would add significantly to the interpretation of biochemical results and would support the theory of biochemical homogeneity of morphologically heterogeneous organelles.

In the present investigation we have studied the activity and localization of acid phosphatase and aryl sulfatase in hepatic parenchymal and renal proximal tubule cells during autolysis and compared the fine structural alterations with the cytochemical findings. Optimal and uniform preservation of fine structure has been secured by use of thin slices of tissue—approximately 0.5 mm thick—and comparisons of the results of fixation in a protein cross linking agent, glutaraldehyde, in addition to osmium tetroxide.

MATERIAL AND METHODS

Young male Sprague Dawley rats weighing 200–250 gms were sacrificed by decapitation following exsanguination and the carcasses were kept at room temperature (+20 C) for periods of 1, 2, 4, 8, 16 and 24 hours. At the appropriate time point the abdomens were opened and slices of liver and renal cortex, approximately 0.5 mm thick, were immersed for 3 hours in 3 per cent purified glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at +4 C. Tissues from normal rats fixed immediately after death served as controls and were treated in exactly the same manner as the ones from experimental animals. Following fixation, tissues were washed at +4 C in 0.1 M tris maleate buffer (pH 7.4) containing 7.5 per cent sucrose for 20–24 hours.

For the demonstration of aryl sulfatase, approximately 6 µ thick free floating frozen sections of glutaraldehyde fixed tissues were incubated in a medium prepared as follows: Thirty mg p-nitrocatechol sulfate (Sigma) was dissolved in 5.0 ml Veronal acetate buffer, pH 5.4. Lead nitrate (0.16 ml of a 24 per cent solution) was added and the medium was adjusted to pH 5.4. A heavy precipitate which was formed when lead was added was removed by filtering the substrate solution. Incubations were performed at +37 C and incubation times were: for renal cortex, 30 minutes, and for liver, 60 minutes. Sections incubated in substrate free media served as controls. Following incubation, sections were washed in 3 changes of 0.1 M acetate buffer, pH 5.0, and were immersed briefly in dilute ammonium sulfide.

For the demonstration of acid phosphatase, approximately 6 µ thick frozen sections were incubated in a Gomori type medium as previously described (17). Incubation times were: for kidney, 10 minutes, and for liver, 45 minutes. Sections incubated in appropriate substrates for aryl sulfatase and acid phosphatase and immersed in ammonium sulfide were mounted on glass slides in glycerine gelatin; some sections were stained with methyl green.

Tissues from normal liver and renal cortex, and from liver and renal cortex subjected to a 24 hour period of "autolysis" were fixed for fine structural observations. Slices of tissue were processed for demonstration of acid phosphatase and aryl sulfatase as described above, with the exception that the frozen sections were cut at 50 µ and the final step—immersion in ammonium sulfide—was avoided. The tissues were postfixed in osmium tetroxide (OsO_4) and processed for electron microscopy as indicated below.

For the fine structural studies of exclusively OsO_4 fixed tissue, small cubes of liver and outer renal cortex were immersed in 2 per cent OsO_4 buffered with s-collidine (pH 7.4) for 1½–2 hours at +4 C. Dehydration and embedding in Epon was performed as previously described (15, 18). Thin sections were cut on LKB Ultrotomes and were stained with lead hydroxide, aqueous uranyl acetate or a combination of lead hydroxide and uranyl acetate; they were studied in a Siemens Elmiskop I electron microscope. Thicker sections cut at 1 µ of Epon embedded tissues were stained with toluidine blue and were studied in the light microscope. When blocks of osmium fixed tissues were studied, the specimens were trimmed in such a way that only the peripheral portions were included for thin sectioning.

RESULTS

I Gross and Light Microscopy

Gross Livers and kidneys appeared moderately enlarged and swollen in animals left in room temperature for 24 hours The cut surfaces were pale at this time and the tissues were soft and friable Similar although less pronounced alterations were noted at earlier intervals after sacrifice and were first noticeable at 4 hours

Light microscopy In sections of glutaraldehyde-fixed hepatic parenchymal and renal cortical cells embedded in paraffin very slight alterations were noted at 24 hours These consisted in cytoplasmic swelling and an increased prominence of cytoplasmic "granules presumed to represent mitochondria In the cells of the proximal and distal convoluted tubules the normally slender and elongated mitochondria seemed to become shorter and take on a more rounded shape This alteration was more clearly revealed in sections of Epon-embedded tissues stained with toluidine blue (29) All the proximal tubules appeared collapsed with obliterated lumens

II Histochemistry

A Liver

1 *Acid phosphatase* In hepatic parenchymal cells of control animals activity was confined to small droplets (up to 1 μ in diameter) which usually showed the typical pericanalicular arrangement (Fig 1) No alteration in the quantity appearance or distribution of these droplets was noted at any interval up to 24 hours (Fig 2)

2 *Aryl sulfatase* As was noted with acid phosphatase reaction product in normal hepatic parenchymal cells was confined to cytoplasmic droplets with a largest diameter of approximately 1 μ With the incubation time employed (1 hour) the number of these droplets appeared to be considerably lower than that of acid phosphatase droplets The aryl sulfatase droplets showed the same localization as the acid phosphatase positive droplets however and were mainly confined to peribiliary regions The appearance quantity and intracellular distribution of aryl sulfatase droplets at 24 hours (Fig 7) was inseparable from that of controls

B Proximal Convoluted Tubules

1 *Acid phosphatase* In control specimens the reaction product was precipitated in cytoplasmic droplets (15 18) (see Fig 3) No alterations were seen in experimental animals (compare Fig 3 with Fig 4)

2 *Aryl sulfatase* Reactive sites were in the form of droplets with a size and distribution corresponding to that of the acid phosphatase droplets (see Fig 5) In addition moderate numbers of very small droplets (0 2–0 4 μ in diameter) were observed in some proximal tubu

stration of two lysosomal enzymes in the same organelle would add significantly to the interpretation of biochemical results and would support the theory of biochemical homogeneity of morphologically heterogeneous organelles

In the present investigation we have studied the activity and localization of acid phosphatase and aryl sulfatase in hepatic parenchymal and renal proximal tubule cells during autolysis and compared the fine structural alterations with the cytochemical findings Optimal and uniform preservation of fine structure has been secured by use of thin slices of tissue—approximately 0.5 mm thick—and comparisons of the results of fixation in a protein cross linking agent glutaraldehyde in addition to osmium tetroxide

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lar cells At 24 hours the distribution and activity of aryl sulfatase appeared to be similar to that in controls

Sections from control and experimental animals incubated in media lacking the substrates (β glycerophosphate or p nitrocatechol sulfate) lacked evidence of precipitation of reaction product

III Electron Microscopy

A Controls

The appearance of hepatic parenchymal and renal proximal tubule cells in control animals was in accordance with previous observations (7 12 15–18 28 36–39)

B Experimental Animals

1 *Hepatic parenchymal cells* Within the cytoplasm fine structural alterations were noted in mitochondria microbodies endoplasmic reticulum and cytoplasmic ground substance (Fig 9) These alterations were diffusely distributed within the cells They were of similar appearance in osmium fixed and glutaraldehyde fixed tissues Many mitochondria appeared more rounded than in controls and all of them lacked matrical granules The matrix was of uniform density and alterations of cristae or the envelope were only rarely encountered The rough surfaced endoplasmic reticulum showed irregular widening of the cisternae which sometimes revealed focal loss of ribosomes Polysomes were not encountered Smooth surfaced endoplasmic reticulum tended to break up into vesicular elements Microbodies showed pallor of their matrix but otherwise appeared normal The cytoplasmic matrix showed focal areas of pallor and appeared to be more voluminous than in controls with cytoplasmic organelles widely separated from one another

Contrasting to these changes was the unaltered appearance of cytosomes (Fig 10) Although occasional cytosegresomes (autophagic vacuoles) were observed these were not more frequent than in controls

2 *Proximal convoluted tubules* For a description of the fine struc

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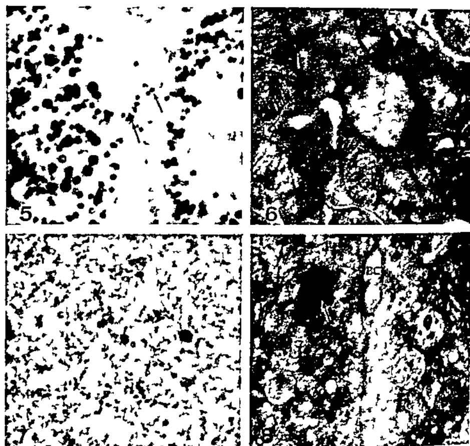
Figs 1–4

Fig 1 Liver control anim l Discrete droplets ma l a ty of bile capill es ×

Fig 2 Liver 24 h urs afte acrifice Droplets of sim l r app an n Fig 1 are presen n peri alicular areas × 75

Fig 3 kidney control ani al Abundant large rop t convoluted tubules A glomerulu ontains a fes small r tl 4 0

Fig 4 kidney 24 hours aft sacrifice Acid pl osphat e le imilar to the ones sho n Fig 3 present in proximal cor olute bul s × 780



Figs 5–8

Fig 5 Kidney control animal Section incubated for the demonstration of aryl sulfatase Activity in proximal convoluted tubules is in the form of large droplets similar to the ones in the acid phosphatase procedure Note the small droplets (*arrows*) which probably are located in distal tubules or collecting ducts × 800

Fig 6 Kidney control animal Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing localization of final product along the margins of cytosome (C) in a proximal convoluted tubule cell × 19 400

Fig 7 Liver 24 hours after sacrifice Section incubated for the demonstration of aryl sulfatase Activity is confined to small discrete droplets which are less numerous than the acid phosphatase droplets (*cf* Fig 2) but appear to show the same localization around bile capillaries × 580

Fig 8 Liver 24 hours after sacrifice Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing a cytosome (*C*) with final product precipitated along the periphery Note absence of reaction product in two microbodies (*Mb*) *BC* bile capillary × 18 000

ture of the normal proximal tubule cells see previous publications (15 16 18)

In the experimental animals alterations of mitochondria were more prominent than in hepatic parenchymal cells (Fig 11) Thus the tendency to attain rounded forms (29) was more apparent and alterations

of the matrix substance with focal pallor were evident Matrical granules were not observed The cristae were numerous Often long cristae connected one side of the envelope with the other Such cristae appeared to be much longer than in the controls While dilatation and fragmentation of the endoplasmic reticulum was frequently observed increased area of cytoplasmic ground substance was not in evidence As in hepatic parenchymal cells cytosomes appeared unaltered

IV *Ultrastructural Cytochemistry*

A *Acid Phosphatase*

In normal hepatic parenchymal and proximal tubule cells the reaction product is localized in cytosomes cytosegresomes and on occasional Golgi membranes (15 18) In accordance with the light microscopic observations a similar localization was noted in the experimental animals in the present study

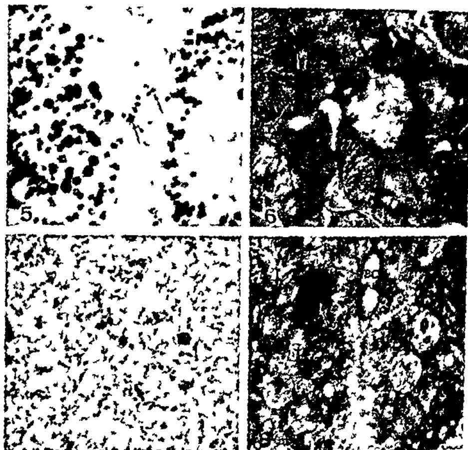
B *Aryl Sulfatase*

In the cells of the proximal tubules of *control animals* reaction product was precipited in the cytosomes (Fig 6) Often the final product appeared as a narrow rim of precipitate overlying the membrane limiting the cytosomes Although occasional cytosomes lacked reaction product precipitation in relation to cytosomes appeared to be the rule Observations are presently too few to permit definitive conclusions concerning the possible occurrence of aryl sulfatase in the Golgi apparatus and cytosegresomes Likewise the nature of the very small reactive sites seen in the light microscope is not clear Microbodies did not seem to contain final product In hepatic parenchymal cells some cytosomes contained reaction product

As demonstrated in Fig 8 reaction product was confined to cytosomes and cytosegresome like bodies in the *experimental animals* Spreading of reaction product was not observed

DISCUSSION

At the present state of our knowledge it is most convenient to base a definition of cellular death upon the notion of irreversibility (26) The time of cell death can accordingly be identified with the time at which the point of no return is reached In the pre ent [illegible] mple system for autolysis cellular death is probably the result of the combined effects of anoxia and lack of exogenous and endogenous substrates Studies of ischemia by v[illegible]ious authors (2 5 26) have indicated that hepatic parenchymal ce[illegible] can tolerate 30 to 4[illegible] [illegible]utes of [illegible] ischemia without reaching the point of no retu[illegible] The [illegible] ons observed 24 hours after [illegible]rifice of the experimental anim[illegible] apparently represent alteration[illegible] [illegible]r beyond those at the point of [illegible] death and are repre[illegible]ntative of a[illegible]olysis



Figs 5-8

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Fig 6 Kidney control animal Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing localization of final product along the margins of cytosome (C) in a proximal convoluted tubule cell × 19 400

Fig 7 Liver 24 hours after sacrifice Section incubated for the demonstration of aryl sulfatase Activity is confined to small discrete droplets which are less numerous than the acid phosphatase droplets (*cf* *Fig* 2) but appear to show the same localization around bile capillaries × 580

Fig 8 Liver 24 hours after sacrifice Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing a cytosome (*C*) with final product precipitated along the periphery Note absence of reaction product in two microbodies (*Mb*) *BC* bile capillary × 18 000

ture of the normal proximal tubule cells see previous publications (1, 16 18)

In the experimental animals alterations of mitochondria were more prominent than in hepatic parenchymal cells (Fig 11) Thus the tendency to attain rounded forms (29) was more apparent and alt rations

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THREE DANISH CASES OF MALIGNANT CHILDHOOD LYMPHOMA (BURKITT)

By

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Received 27 ix 66

In 1958 *Burkitt* pointed out that a characteristic jaw sarcoma often seen in children in Uganda where it originally had been described in 1904 by Cook was part of a multifocal tumour syndrome affecting abdominal viscera *O Connor & Davies* added the information that jaw and abdominal tumours of identical lymphomatous structure con stituted about half of alle malignant childhood tumours registered in the Kampala Cancer Registry Uganda

When in 1961 *O Connor* reviewed 106 cases including 28 examined post mortem he found the African lymphoma a pathological entity but classified the vast majority or 83 per cent of cases as a poorly differ entiated malignant lymphoma of lymphocytic type while the rest were designated as stem cell histiocytic and mixed lymphocytic histiocytic lymphomas *Hull & Wright* (1963) however drew the limits somewhat more narrowly They found the presence of multifocal jaw tumours in a child the most certain clinical criterion and the combination of a single jaw tumour with tumour in the thyroid kidneys or ovaries or extradural spinal deposits causing paraplegia they considered virtually diagnostic of this tumour syndrome However they found that all cases should be biopsied and that only those conforming to defined histological criteria should be included Histologically they found that the tumour always is a poorly differentiated lymphocytic (lympho blastic) lymphoma containing a variable admixture of non malignant histiocytes

Wright (1963) on the basis of the Kampala material described the tumours as composed of sheets of round cells with round or reniform nuclei a well defined nuclear membrane and one to fi[illegible] often in conspicuous eosinophilic nucleoli with a narrow [illegible]m of a[illegible]ph[illegible]lophilic cytoplasm Interpersed between the lymphoid [illegible] are n[illegible] histio cytes usually with abundant clear or vacuol[illegible] and often actively phagocytic containing large an[illegible] nuclear deebris These cells do not have the char[illegible] of m[illegible]lignant cells and give the histological picture a charac[illegible] feature which however is not constant Its pro[illegible] *Wright* varies

with different fixation, sectioning and staining procedures and with the type of tissue being invaded. Post mortem loosening and separation of cells lessens its prominence in certain tissues.

Wright (1964) reports that occasionally leukaemia occurs as a terminal manifestation of the disease, and it seems interesting that while leukaemia in Denmark amounts to about half of all malignant diseases in childhood (*Clemmesen* 1965) it is very rare in Africa (*Davies* 1958). It follows from local conditions that estimates of incidence, not to speak of age distribution, will be approximate only in those regions, but *Burkitt* (1963) on the basis of 144 cases estimated incidence of the childhood lymphoma in Uganda at 1 per 50–100 000, with local findings ranging up to 6 among a population of about 100 000. From case finding studies he found the disease limited to a belt across Africa limited in the North by the Sahara and towards the South by a border line through Angola, traversing the southern tip of Congo, excluding Zambia and Rhodesia but including Tanzania and Malawi. According to *Burkitt* the lymphosarcoma is extremely rare in the highlands of Kenya and Ruanda Burundi and he suggests that the distribution of the tumour depends on humidity and vegetation, explainable by the further assumption of an arthropod vector. Apparently the absence of the syndrome in the Kinshasa (Leopoldville) area is out of harmony with local humidity and vegetation, but is explained as due to an energetic extermination of mosquitos in that area.

Cases resembling those published from Central Africa have during later years been published from various parts of the world. From South Africa *Glucksman* (1963) reported four cases in white and four in non white children, and single cases as well as series have been published from the United Kingdom (*Wright* 1964), the United States (*O'Connor et al* 1965, *Dorfman* 1965), from Papua and New Guinea (*ten Seldam et al* 1966) and Columbia, South America (*Beltran* 1966). Up to now no cases seem to have been reported from the European continent and since a recent case in this institute turned our attention to the likelihood of similar cases, records were reviewed for autopsy cases presenting the typical histological features.

CASES

Among post mortems from the years 1955 to Aug. 1966, representing a total of 1615 autopsies, 36 had been performed on persons aged below 20 years and suffering from diseases of the lymphatic and reticular system. Besides the recent case of a boy diagnosed from biopsy, two autopsy records for girls showed the histologic features characteristic of *Burkitt's* syndrome.

Case 1

Thirteen year old daughter of fitter. For a couple of years repeated catarrhal infections treated with tonsillectomy. For two months before admission swelling in

TABLE 1

Clinical Findings

Case No	Sex	Age	Site of primary	Peripheral lymphnodes	White cell count	Total duration of illness	Treatment
1 167/59	♀	13	Right parotic	not involved	3 700	4 months	Operation & X rays
2– 15/66	♀	9	Extra dural spinal canal	not involved	7 300	1 month	Operation
3– 7?/66	♂	4	Left lat cerv reg	not involved	5 900	2 months	X rays

TABLE 2

Involvement of Organs in 3 Autopsy Cases

	Case 1	Case 2	Case 3
Skin	—	—	—
Tonsils	—	—	+
Neck	—	+	+
Mediastinal Glands	+	+	+
Retroperitoneal lymphglands	—	+	+
Lungs	—	+	+
Heart	+	—	—
Stomach	+	+	+
Small Intestine	+	—	—
Bowel & Rectum	—	—	—
Liver	—	—	—
Spleen	—	—	+
Pancreas	+	+	—
Supra Renal Glands	—	+	—
Kidney	+	—	+
Ovaries/Testis	+	+	—
Bone Marrow	+	+	+
Spinal Canal		+	
Thyroid	—	+	—

the right side of the neck, rapidly increasing during the last week On admission a tumour of the right parotid gland me suring ca 3 × 5 × 5 cm Diffe ential count normal. Toxoplasmosis sero reaction negative Partial removal of tumour was followed by application of 3330 R Death six weeks after admission

Post mortem S ars of right parotid reg on Enlargement of lymph nodes of mediastinum measuring up to 2 × 3 cm and nodular infiltrations of pericard um gastric and intestinal muco a of up to 2 cm size Ovaries measured 9 × [illegible] × [illegible] and pancreas and kidneys showed considerable infiltration with enla [illegible] Spleen and bone marrow showed histologically demonstrable infiltration

Case 2

Nine year old daughter of cabinet maker Adm [illegible] by [illegible] development of medullary transection syndrome An e [illegible] rresponding to the level of 4th to 9th thoracic verteb a was [illegible] oved b t the patient died two weeks later

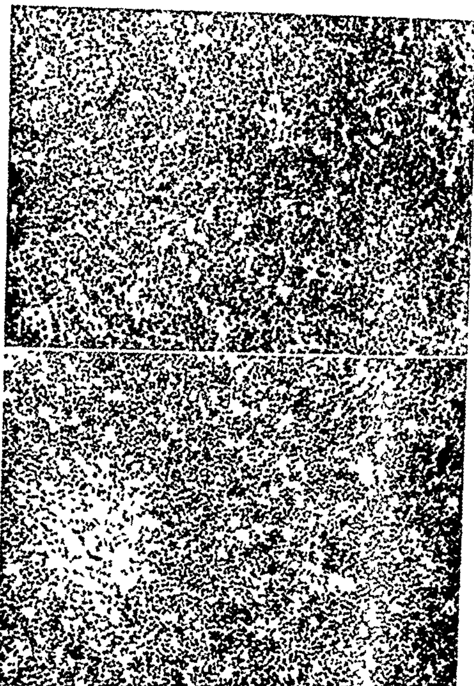


Fig 1 Case 1 Primary tumor in parotis (H + E, × 80)
Fig 2 Case 2 Tumorinfiltration in pancreas with preservation of "starry sky" appearence (H + E, × 80)

Post mortem showed lymphosarcoma of the thoraco lumbal medulla (large) epidural with secondary myelomalacia (Histologist Erna Christensen) Lymph nodes of neck mediastinum and along the abdominal aorta were enlarged measuring up to 7 cm Extensive infiltration of tumour tissue was found in the thyroid the

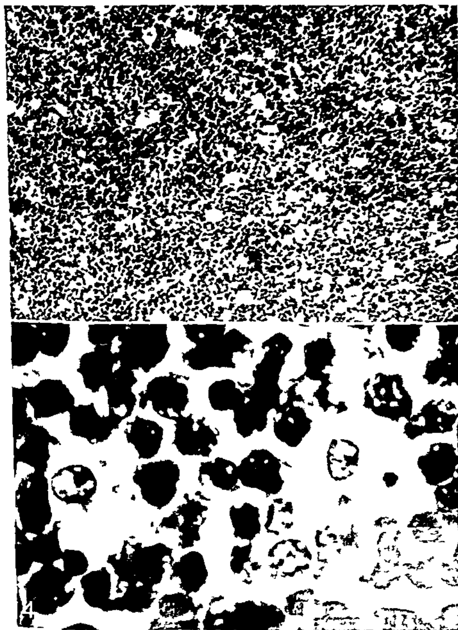


Fig 3 Case 3 Primary tumor in lymph node in right ce i l on I + E × 80)
Fig 4 Case 3 Detail of Fig 3 showing phagocyti c , th pal n leus and abundant ill defined c toplasm The scattering of th cells in tissue constitutes th starry sky appearence (H + E × 800)

lungs pancreas and surroundings and adrenals Gastric mucosa showed nodular infiltrations ovaries measured 8 × 4 × 2 cm infiltrated with tumour tissue There was gross infiltration of bone marrow and microscopic infiltration of the spleen No visible enlargement of axillary and inguinal lymph nodes

Case 3

Four year old son of water and gasfitter The family never left Denmark except for the mother s visit to Italy some six months before the birth of the patient Six months before admission the father and the son developed "influenza" One month before admission a node was noticed on the left side of the neck persisting in spite of treatment with penicillin and measuring 7 × 4 × 5 cm located with its upper pole levelling with the mandibular angle and its anterior limit coinciding with that of the sternocleid muscle A soft microadenitis of the accessorius chain was noticeable but no other nodes discernable Toxoplasmosis sero reaction negative After treatment with 1075 R the patient died two weeks after admission

Post mortem examination showed the enlarged lymph nodes on the neck as described Further enlarged nodes along the aorta and in the mesenters measured up to 2 × 3 cm Infiltrates of tumour tissue were found in the columna and in both kidneys

A survey of clinical and pathological findings is given in Tables 1 and 2

The histological findings of the three cases were all alike and in full correspondance with the description given by *Wright* Within the limitations of practical pathology the cases must then be considered as indistinguishable from those described as Burkitt s tumour It is true that in Africa the disease is rarely reported to have been observed first in one of the palpable lymph nodes like in our case 3 but this can hardly be considered a decisive criterion

The histological pictures have been illustrated by photomicrographs 1–4

It remains an open question to which extent the demonstration of scattered reticulum cells in a childhood lymphosarcoma—the socalled starry sky appearance will entitle us to assume a clinical entity but it does seem more likely than not that the post mortem findings in the present three cases and perhaps also their lack of radiosensivity will designate them as belonging to the group of childhood lymphosarcomas often termed Burkitt s tumour

SUMMARY

Report on three cases of childhood lymphosarcoma with histologic and autoptic resemblance to Burkitt s African lymphosarcoma in children diagnosed in Denmark

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AN IMPROVED METHOD FOR THE HISTOCHEMICAL DEMONSTRATION OF PHOSPHORYLASE IN TISSUE SECTIONS

By

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The principles for the histochemical demonstration of phosphorylase activity in tissue sections were first established by *Takeuchi & Kuriaki* (4) Unfixed cryostat sections were incubated in a medium containing glucose 1 phosphate and the co factor muscle adenylic acid Tissue components possessing sufficient phosphorylase activity synthesised iodophilic polysaccharides from glucose 1 phosphate and after incubation the sites of phosphorylase activity were visualized by simple iodine staining

The sensitivity and the exactness of localisation in enzymatic histochemical procedures greatly depend on the tendency of the enzyme and the reaction product to diffuse during incubation and in the case of the phosphorylase reaction the conditions are particularly unfavourable the enzyme is localized in the ground substance of the cytoplasm and the reaction product is a water soluble polysaccharide To diminish this difficulty *Takeuchi* recommended the inclusion of ethanol (20 per cent v/v) in the incubating medium (3 4) and later *Franko & Pallama* advocated the additional inclusion of polyvinyl pyrrolidone (PVP) (7 5 per cent w/v) (1) We found however that these improvements did not give satisfactory demonstration of phosphorylase particularly in gastrointestinal epithelium and it was therefore decided to determine the optimum concentrations of protective agents in the phosphorylase medium

MATERIAL AND METHODS

Biopsies of human tissues were taken during endoscopy (rectal mucosa) percutaneously (liver) or during operations (skin striated muscle colonic mucosa colonic and rectal tumours) The biopsies were placed on thin discs of cork and quenched by immersion in liquid nitrogen Sections were cut at 8 10 µ using a SLEE cryostat All incubating media described below were tested on nine rectal biopsies (from patients suffering from irritable colon or ulcerative colitis) one rectal polyp one liver biopsy four skin biopsies and four muscle biopsies Incubating medium C (see below) was tested on a greater number of tissue specimens

Incubating media Nine different incubating media (labelled A to I) were prepared, all containing 50 mg of glucose 1 phosphate 5 mg of muscle adenylic acid 1 mg of

glycogen and 9 mg of sodium fluoride in 5 ml of 0 1 M acetate buffer (pH 6 0) The concentrations are those recommended by Eränkö & Palkama (1) To media A B and C were added no ethanol and 0 0 5 and 1 0 g of polyvinyl pyrrolidone (PVP) (average MW = 12000 obtained from Koch Light Ltd) respectively To media D E and F were added 0 5 ml of ethanol and 0 0 5 and 1 0 g of PVP respectively To media G H and I were added 1 0 ml of ethanol and 0 0 5 and 1 0 g of PVP respectively (Table 1) When stored at 4 C the media were stable for at least one week. The media were preheated to room temperature before use



Fig 1

Histochemical procedure 1) Mounting of frozen sections on slides and air drying for 5 minutes 2) Incubation for one hour at 37 C under conditions suitable to prevent evaporation For this purpose a slide with a central shallow concavity was used The concavity was filled with incubating medium and the slide with the section was placed on top of the concavity thus forming a closed incubation chamber (Fig 1) Air bubbles were avoided 3) Removal of slide with section and drying in air 4) Rinsing in 40 per cent ethanol for 2 minutes 5) Air drying 6) Fixation in absolute ethanol for 5 minutes 7) Air drying 8) Iodine staining One or two drops of glycerol iodine (equal parts of Gram s iodine solution and glycerol) were placed on the section After a few minutes a coverslip was mounted on top The iodine staining was stable for several hours

Control experiments Sections were treated with an incubating medium from which glucose 1 phosphate had been omitted

Evaluation The staining intensity of the various tissue components in the preparations were roughly graded according to an arbitrary scale ranging from (+) to ++++ The preparations were coded so that it was not known during microscopy of a preparation which incubating medium had been used

RESULTS

The polysaccharides which were synthesised in the tissue sections during incubation stained violet or blue with iodine The staining intensity was recorded and the more important results are summarized in Table 1 The content of protective agents in the incubating medium greatly influenced the outcome of the phosphorylase reaction Phosphorylase activity was only demonstrated satisfactorily in rectal epithelium when the incubating medium had a high concentration of PVP (media C and F) whereas an additional high concentration of ethanol inhibited the reaction (medium I) When medium C was used the surface and crypt epithelium presented a strong staining (Fig 2 c and d) All other media gave unsatisfactory results including G and H which as recommended in previous works (1 3 4) had a high ethanol concentration but contained little or no PVP Using those media the sections were either unstained or presented weak staining as depicted in Fig 2 a and b

It was also noticeable that sections incubated in media with a high PVP content were very well preserved whereas the other media damaged the tissue during incubation to a varying degree

A high PVP content also improved the phosphorylase reaction in



Fig ?

a and b—Weak staining reactions in crypts of Lieberkühn and a rectal polyp after incubation in phosphorylase media C and H. c and d—Intense staining reactions in the same structures after incubation in phosphorylase medium C.

TABLE 1

The Staining Intensity Obtained Using Incubating Media with Different Ethanol and Polyvinyl Pyrrolidone (PVP) Contents Has Been Graded According to an Arbitrary Scale

Incubating medium			*Staining intensity*			
			Rectal mucosa			
Medium	Ethanol (ml added to ml medium)	PVP (g added to 5 ml medium)	Crypts of Lieberkühn	Muscularis mucosae	Epithelium	Striated muscle
A	0	0	(+)	(+)	+	+++
B	0	0.5	+	+	+	++++
C	0	1.0	+++	+++	++	++++
D	0.5	0	(+)	(+)	+	+++
E	0.5	0.5	++	+++	+	++++
F	0.5	1.0	+++	+++	++	++++
G	1.0	0	(+)	(+)	+	+++
H	1.0	0.5	+	++	+	++++
I	1.0	1.0	+	++	+	+++

(+) weak or no staining + week staining ++ moderate staining +++ strong staining ++++ intense staining

smooth muscle epidermis and liver cells whereas satisfactory staining of striated muscle was obtained using any of the media

To test the reproducibility of the satisfactory results which were obtained using *incubating medium C a greater number of tissue specimens* were examined using this medium only In all cases the results were comparable to those described

Control preparations incubated in media without glucose 1 phosphate showed no staining with iodine

DISCUSSION

In the histochemical experiment phosphorylase in conjunction with various branching factors catalyzes the synthesis of iodophilic polysaccharides from glucose 1 phosphate whereas phosphorylase *in vivo is mainly concerned with the opposite process i e degradation of glycogen* to glucose 1 phosphate (2) The enzyme thus plays an important role in glycogen metabolism

The present experiments show that the histochemical demonstration of phosphorylase in some tissue structures may be greatly improved by increasing the viscosity of the incubating medium through the addition of a larger quantity of PVP (200 mg per ml) This modification resulted in stronger staining reactions in a number of tissue structures presumably by reducing the diffusion of polysaccharides into the medium and at the same time it prevented damage to the tissue sections during incubation The modification also permitted the use of thin sections (8–10 μ) instead of sections of 40 μ as recommended by *Takeuchi & Kuriaki* (4) Addition of ethanol to the medium was not found to be advantageous in these experiments but is essential for the detection of phosphorylase in blood films (5)

It is envisaged that the use of high concentrations of PVP may also *prove of* value in other histochemical enzyme reactions

SUMMARY

The histochemical demonstration of phosphorylase in tissue sections is hampered by the fact that the end product of the reaction is water soluble If however the viscosity of the incubating medium is increased by addition of a larger quantity of polyvinyl pyrrolidone than usually recommended the demonstration of the enzyme is greatl[illegible] Additional inclusion of ethanol in the medium only [illegible] th[illegible]ct on This modification is particularly valuable for th[illegible] of phosphorylase in re[illegible]l epithelium

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Control mice. Ovaries of untreated control mice of the same strain and age were examined and compared with the ovaries of mice in the 3 experimental groups.

All mice were weaned at the age of 4–5 weeks and housed 2–5 mice in a cage until sacrifice. Mice were killed and their ovaries examined at different time intervals after DMBA treatment. The ovaries were fixed in Bouins solution, imbedded in paraffine, serially sectioned at 5 µ and stained in haematoxylin eosin. The pathology of the ovaries was studied and differential counts of the oocytes were done (13). The oocytes were divided into two main groups: 1) The small oocytes, i.e. 20 µ or less in diameter. 2) The growing and large oocyte, i.e. those germ cells that have begun to grow and have a diameter of more than 20 µ as well as those that have reached their maximum size of 60 µ (13).

RESULTS

Oocyte Counts

The oocyte counts of all three experimental groups are given in Table 1. The numbers of oocytes counted in the ovaries of DMBA treated mice were expressed as a percentage of oocytes in the ovaries of not treated control mice of the same age.

TABLE 1

Surviving Oocytes at Different Ages after Treatment with DMBA by Different Routes

	Group I (Street) (direct ovary painting)		Group II (Bagg) (Feeding)		Group III (Bagg) (intraperitoneal injection)	
	Small oocytes %	Growing and large oocytes %	Small oocytes %	Growing and large oocytes %	Small oocytes %	Growing and large oocytes %
28 days			63	100	15	43
35 days	57	97				
42 days			4	58	0	17
49 days	33	77				
3 months	6	28	0.7	10	0	0
6 months	0.2	1.4	0	0	0	0
9 months	0	0.5				
12 months	0	0				

Group I (direct ovary painting). In the ovaries examined 14 days after the organs were painted with DMBA almost one half of the small oocytes were destroyed. At the age of 49 days (28 days after painting) only one third of the small oocytes survived. At the age of 6 months the ovaries contained between 0 and 7 small oocytes. A normal 6 months old Street mouse had 950 small oocytes in one ovary. After the age of 6 months no small oocytes survived in the DMBA painted ovaries.

The growing and large oocytes were also destroyed but at a slower rate. Ovaries of 4 mice were examined at the age of 9 months. These contained between 0 and 4 growing and large oocytes. A normal Street mouse aged 9 months had 300 growing and large oocytes in one ovary

Group II (feeding) The reduction in the number of oocytes in this group occurred faster than in the previous one At the age of 42 days (21 days after feeding of DMBA) only 4 per cent of the small oocytes survived In group I the same proportion of surviving small oocytes was not reached until the age of 3 months *i e* 70 days after DMBA-painting of the ovaries In group II small oocytes were not observed after the age of 3 months As in group I the growing and large oocytes were destroyed at a lower rate than the small ones at the age of 42 days about one half survived at 3 months 10 per cent were still found however after this age none were found any more

Group III (intraperitoneal injection) A very rapid destruction of oocytes occurred in this group Already 7 days after intraperitoneal injection of DMBA (age 28 days) only 15 per cent of the normal complement of small oocytes were still present At the age of 42 days (21 days after injection) the ovaries were depleted of small oocytes while about one fifth of the growing and large oocytes survived A normal Bagg mouse aged 42 days had 5000 small and 320 growing and large oocytes in one ovary At the age of 3 months no more oocytes were present in the ovaries of the DMBA injected mice

Pathology

The histologic changes in the first 2 months after treatment with DMBA were insignificant except for the reduction of oocytes in all stages of follicle development

Group I (direct ovary painting) At the age of 3 months the histologic structure of the ovaries was still dominated by follicles in different stages of development although their number was reduced Compared with the control ovaries however a larger number of degenerated follicles was noted and the number of old corpora lutea was increased The amount of stroma was markedly increased this was luteinized and formed groups and cords throughout the ovaries Rings of follicle cells without oocytes (empty rings) formed a rim just beneath the surface epithelium in one of the ovaries examined

At the age of 6 months the histologic picture was no longer dominated by developing and ripe follicles Just beneath the surface epithelium which showed a few down growths a rim of empty rings and small solid balls of follicle cells surrounded by a layer of flattened theca cells (pseudo follicles) was seen Some degenerated follicles were present but an accumulation of corpora lutea some of which showed a tendency of merging occupied most of the organ The stroma was luteinized

Unilateral non infiltrating ovarian tumours were found in two mice out of four at the age of 9 months These were luteomas they consisted of nodules of lutein cells The ovaries of mice that had not developed tumours at this age showed the same pathologic picture as seen at 6 months

4 mice were examined at the age of 12 months. They had all unilateral ovarian tumours: 3 were luteomas showing growth into the periovarian fat tissue, while one was a big granulosa cell tumour.

Group II (feeding). At the age of 3 months the cortex of the ovaries showed empty rings and pseudo follicles as well as a few normal and degenerating follicle. The center of the ovaries was mainly composed of well defined or merging corpora lutea (in one single section as many as 14 different corpora lutea could be counted) and central cords of luteinized stroma.

At the age of 6 months two mice out of two had a unilateral granulosa cell ovarian tumour.

Group III (intraperitoneal injection). At the age of 3 months the ovary contained only few empty rings and pseudo follicles; no follicles normal or degenerating were found. Almost the entire organ was composed of merging corpora lutea, *i.e.* one mass of corpus luteum material in which separate corpora lutea could not be identified. Stroma was not present.

At the age of 6 months unilateral granulosa cell tumours were found in two out of two mice. Neither oocytes nor rests of follicles or corpora lutea or any other constituent of a normal ovary were recognized in these ovarian tumours.

DISCUSSION

Tumour Incidence

The number of experimental animals in the present material is too small for statistical evaluation. Nevertheless the results show an early development and a high incidence of ovarian tumours. These results were obtained using only a single application and a smaller total dose of DMBA than has been used before. In *group I* the ovaries were painted directly with a 0.5 per cent solution of DMBA. Though the absolute dose of DMBA that was applied cannot be calculated, it is presumably less than 0.25 mg of DMBA. In this group half of the animals had developed ovarian tumours at the age of 9 months, and all the animals had ovarian tumours at the age of 12 months. In *group II* 0.25 mg DMBA was fed to the animals, and both mice at the age of 6 months had ovarian tumours. In *group III* 0.25 mg of DMBA was injected intraperitoneally to the animals, and also in this group ovarian tumours were already found in both mice aged 6 months.

Marchant (11) painted the skin of mice aged 2½ to 4 months 6 times at fortnightly intervals, the total dose of DMBA being 6 mg. 7 months after first skinpainting she found 70 per cent of the mice with predominantly unilateral ovarian tumours.

Biancifiori et al. (1) fed 2½ to 4 months old mice 8 times at weekly intervals, the total dose of DMBA being 8 mg. During the entire observation period, which extended over 9 months, 68 per cent of the mice had developed ovarian tumours.

In his investigations on the development of spontaneous tumours in mice *Lefevre* (9) found a maximum of 27 per cent ovarian tumours in 9 to 11 months old mice. In that experiment mice aged 1½ months were skinpainted once weekly for 24 weeks. The total dose of DMBA has not been reported.

The high tumour incidence in the present experiment may be explained in three different ways.

First, the strains of mice used by the author (*Street & Bagg*) may be especially susceptible to the development of ovarian tumours after treatment with DMBA. It is known that different strains of mice have a different sensitivity to tumour development after treatment with carcinogenic chemicals (1, 4).

Second, the routes of applying the DMBA may be of importance for the development and the incidence of the induced tumours. Direct ovary painting with DMBA followed by development of ovarian tumours has not been reported earlier. *Lefevre* (9) found 2 ovarian carcinomas in mice aged 8 months following intraperitoneal injection of DMBA, but the observation was not followed up, and the age of the mice at the time of injection and the dose of DMBA used is not reported.

It could be expected that direct ovary painting or intraperitoneal injection of DMBA would result in a higher tumour incidence than other routes of application, as the carcinogen in those cases comes into more intimate contact with the ovaries. It could also be expected that feeding the DMBA would result in a higher tumour incidence than painting the skin, as the DMBA in the first case is applied internally without any risk of being lost from the organism. Whether the carcinogen after skinpainting is mainly resorbed through the skin or whether it is mainly licked off and consumed is not known, but once applicated on the external hairy surface some of the DMBA may be lost.

However, it is not likely that the different routes of carcinogen application alone are responsible for different incidences of ovarian tumour development for the following reasons. In the skinpainting experiments (11) the total dose of carcinogen were so much larger (6 mg of DMBA) than in the present study (0.25 mg) that the ovaries in spite of the indirect route of application probably have been exposed to at least the same amount of DMBA as after direct ovary painting and intraperitoneal injection. Furthermore, if the present feeding experiment (group II) is compared with the feeding experiment of *Biancifiori et al.* (1) it is seen that although the routes of application are the same, the present study has resulted in a higher tumour incidence—in spite of the fact that *Bianchifiori et al.* (1) fed 32 times as much DMBA (8 mg) as was fed in the present study (0.25 mg). And finally no difference in the tumour incidence was found in the present group II and III (same strains) though the DMBA was applied orally and intraperitoneally respectively.

A third factor influencing the incidence and time of tumour develop-

4 mice were examined at the age of 12 months. They had all unilateral ovarian tumours: 3 were luteomas showing growth into the periovarian fat tissue, while one was a big granulosa cell tumour.

Group II (feeding). At the age of 3 months the cortex of the ovaries showed "*empty rings*" and "*pseudo follicles*" as well as a few normal and degenerating follicles. The center of the ovaries was mainly composed of well defined or merging corpora lutea (in one single section as many as 14 different corpora lutea could be counted) and central cords of luteinized stroma.

At the age of 6 months two mice out of two had a unilateral granulosa cell ovarian tumour.

Group III (intraperitoneal injection). At the age of 3 months the ovary contained only few "empty rings" and "pseudo follicles"; no follicles, normal or degenerating, were found. *Almost the entire organ was composed of merging corpora lutea, i.e. one mass of corpus luteum* material in which separate corpora lutea could not be identified. Stroma was not present.

At the age of 6 months unilateral granulosa cell tumours were found *in two out of two mice. Neither oocytes nor rests of follicles or corpora* lutea or any other constituent of a normal ovary were recognized in these ovarian tumours.

DISCUSSION

Tumour Incidence

The number of experimental animals in the present material is too small for statistical evaluation. Nevertheless the results show an early development and a high incidence of ovarian tumours. *These results were obtained using only a single application and a smaller total dose* of DMBA than has been used before. In *group I* the ovaries were painted directly with a 0.5 per cent solution of DMBA. Though the absolute dose of DMBA that was applied cannot be calculated, it is presumably less than *0.25 mg* of DMBA. In this group half of the animals had developed *ovarian tumours at the age of 9 months*, and all the animals had ovarian tumours at the age of 12 months. In *group II* 0.25 mg DMBA was fed to the animals, and both mice at the age of 6 months had ovarian tumours. In *group III* 0.25 mg of DMBA was *injected intraperitoneally to the animals, and also in this group* ovarian tumours were already found in both mice aged 6 months.

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ment might therefore be found in the age at which the animals are treated with DMBA. In the present experiments the carcinogen was applied to immature mice (at the age of 21 days), while the animals used in *Lefevre's* (9), *Marchant's* (11) and *Biancifiori et al.'s* (1) experiments were mature at the time DMBA treatment was begun (1½ to 4 months of age).

Kaplan (8) found a higher incidence of X-ray induced ovarian tumours when the mice were irradiated at the age of 1 month (immaturity) than when they were irradiated at later ages (in maturity). Within the immature period of life *Peters & Levy* (12) found a maximum radiation sensitivity of mouse ovaries at the age of 21 days.

The age of 21 days was therefore chosen for DMBA treatment, hoping to obtain a high incidence of ovarian tumours, and it was presumed that the damage to the ovaries of the two tumourigenic procedures, i.e. X-irradiation (3, 10) and DMBA treatment might be identical.

Pathology and Oocytes

The pathologic changes in the DMBA treated ovaries which occur before tumour growth is established (the pre-tumourous changes) are: Degeneration of follicles, appearance of empty rings and pseudo-follicles, increase and luteinization of the stroma and persistence and merging of corpora lutea. These changes progress as the number of oocytes regress. From which cellular component(s) the tumour ultimately develops in the DMBA damaged ovary cannot be discussed on basis of the present material.

The fate of the oocytes during ovarian tumourigenesis is only partly known. It has been reported that the follicles (which contain the large oocytes) gradually disappear from the ovaries before tumour growth takes place, whether the stimulus was DMBA treatment (1, 11), X-ray irradiation (3) or transplantation of an ovary to the spleen of a gonadectomized host (2).

In a study on X-ray induced ovarian tumours in mice (6) it was noted that the primary follicles had disappeared from the ovaries 7 days after irradiation of mice aged 3 days. These ovaries developed tumours 9 to 10 months after the irradiation. From the present experiments it appears that a destruction of the small oocytes is also the first recognizable cellular event that occurs in the ovaries after treatment with DMBA. It further appears that the pre-tumour changes do not set in until the small oocytes have been considerably reduced in number. Tumour tissue and oocytes, small or large, were not found together in the same ovary. Consequently, it is likely that a destruction of all oocytes is a condition sine qua non for neoplastic growth in the ovaries. However, whether ovarian neoplasia inevitably will take place merely if the oocytes are destroyed is more uncertain. That this is probable is supported by the observation that all genetically sterile

mice i e mice born with only a few oocytes in their ovaries invariably develop spontaneous ovarian tumours (14)

The mechanism by which the DMBA or its metabolites acts upon the ovaries is still unknown The author has shown that a single application of 0 25 mg of DMBA to 21 days old mice is sufficient to induce ovarian tumours in all treated mice after a latency period of 5 to 8 months it is however not clear whether the carcinogen acts acutely for only a short period of time or whether its action continues until the tumour develops Experiments have been started to determine how and for how long DMBA acts upon the ovaries following a single application

The present study has already contributed to answer the first of these two questions The first event in the ovaries following treatment with DMBA is a rapid destruction of the small oocytes the small oocytes therefore seem to be the primary target cells of DMBA in the ovaries of immature mice

SUMMARY

Immature female mice of two different strains were treated once with solutions of DMBA in olive oil by 3 different routes Oocyte counts were done and histological changes in the ovaries were described at different time intervals after treatment The small oocytes were destroyed first followed by a reduction in the number of the growing and large oocytes A characteristic sequence of pre tumourous pathologic changes in the ovaries was found which preceeded neoplastic growth Ovarian tumours occurred in all mice at 6 months following oral or intraperitoneal application of the DMBA and at 12 months after direct ovary painting with the carcinogen An early development and a high incidence of ovarian tumours was found The possible reasons for this are discussed in relation to 1) different sensitivity to DMBA of different strains of mice used 2) different routes of DMBA application and 3) different sensitivity to DMBA in immature and mature mice It is concluded as neoplastic growth in the ovaries occurs only after all oocytes have been destroyed that the small oocytes are the primary target cells of DMBA in immature mouse ovaries

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AUTORADIOGRAPHIC INVESTIGATIONS OF AMYLOIDOSIS PRODUCED IN MICE BY TRANSPLANTATION OF SPLEEN CELLS FROM CASEIN TREATED SYNGENEIC DONOR MICE

By

POUL RANLOV & OLE WERDELIN

Received 19.xi.66

In a recent report we were able to demonstrate the regular development of spleen amyloidosis in normal C3H mice 2 to 6 days following the intravenous administration of 10^8 spleen cells from syngeneic donors previously hyperimmunized with casein (*Werdelin & Ranlov* 1966). After the cell transfer the recipient mice were treated with nitrogen mustard; serial killings 1, 2, 3, 4 and 5 days after the spleen cell transfer showed no amyloid formation in the recipient spleens until after 48 hours when some of the recipients developed thin incomplete rings in the perifollicular regions of the Malpighian corpuscles. During the following days of nitrogen mustard treatment the incidence and severity of the amyloid lesions increased. At day 5 after the cell transfer all recipient spleens showed marked amyloidosis. In other organs amyloid could not be demonstrated. Control experiments employing 1) the transfer of spleen cells from normal non immunized syngeneic donors followed by nitrogen mustard treatment of the recipients, 2) treatment with nitrogen mustard alone and 3) transfer of spleen cells from donors hyperimmunized with casein but omitting the nitrogen mustard treatment of the recipients, all failed to produce amyloid lesions in the respective recipients. Therefore it was tentatively concluded that the production of amyloid in the recipient spleens depended on two consecutive events: 1) A prolonged casein stimulation of the donor prior to the cell transfer and 2) a suppression of the transferred cells with nitrogen mustard after the transfer, thus illustrating the theory of a local cellular and biphasic pathogenesis of amyloidosis originally put forward by *Teilum* (1964).

In our above cited earlier experiments a prominent feature was the exclusive splenic localization of the resulting amyloid lesions in the

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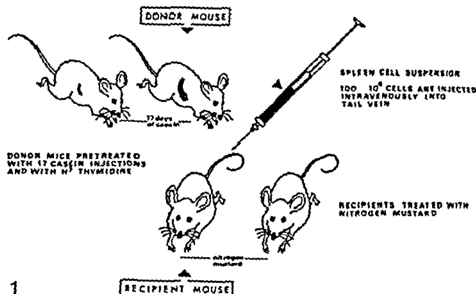


Fig 1
General design of the experiment

recipients In addition the amyloid deposits appeared more cellular than what is usually seen in experimental amyloidosis Based on the assumption that the recipient amyloid was produced and precipitated *in situ* by the transfused spleen cells it was decided under similar conditions to transfuse isotopically labelled spleen cells from casein treated donors to normal syngeneic recipient mice subsequently treated with nitrogen mustard in order to define the route of migration of the donor cells and their possible relationship to the amyloid deposits eventually developing in the recipients

MATERIAL AND METHODS

38 closely inbred C3H mice were used They were aged from 2 to 4 months and the sex distribution was equal They were at random divided into a donor group and a recipient group In Fig 1 the main principle of the experiment is outlined

Donors 12 mice were given a total of 17 subcutaneous injections administered daily as ½ ml of a 5 per cent solution of sodium caseinate 6 times a week On the day after the last injection the donor mice were killed by cervical dislocation and the spleens were harvested and pooled 72 48 and 24 hours before killing each donor mouse received intraperitoneally 0.5 microcurie of H^3 thymidine per g body weight 3 hours before killing the same amount of H^3 thymidine was administered intravenously The H^3 thymidine obtained from The Radiochemical Centre Amersham (U.K.) had a specific activity of 5 000 millicuries per millimol and was dissolved in normal saline so that 0.5 ml contained 10 microcuries of H^3 thymidine

Spleen cell suspension From each donor spleen a small piece was taken for microscopy The rest was pooled in cold Ringer's solution and suspended by gentle manipulation in a loose fitting Potter Elvehjem homogenizer The cell suspension was washed 3 times in cold Ringer's solution It was adjusted so that 0.5 ml contained 10^8 nucleated cells As judged by the trypan blue test the viability ranged from 85 to 90 per cent

Recipients 26 mice received each 10^8 nucleated spleen cells by intravenous injection in the tail 6 mice died within 15 minutes after the injection and these are in the following referred to as group 0 The remaining 20 mice each received a subcutaneous injection of 0 05 mg nitrogen mustard (Frasol®) dissolved in ¼ ml isotonic saline whereupon they were further divided as follows

group I 5 mice killed 24 hours after cell transfer
group II 5 mice killed 2 days after cell transfer
group III 5 mice killed 3 days after cell transfer and
group IV 5 mice killed 4 days after cell transfer

The recipients of the groups III and IV received an additional dose of 0 05 mg nitrogen mustard 48 hours after the cell transfer

The recipients were killed by ether inhalation Tissues were fixed in 10 per cent neutral formalin paraffin embedded and sections were cut 5 microns thick Amyloid was identified by its morphology in haematoxylin eosin and P A S stained sections and by its birefringence with Congo red under crossed polars

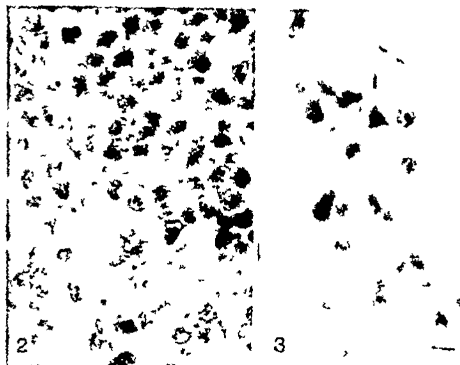
Autoradiography The deparaffinized sections were coated with K Nuclear Research Emulsion (Ilford) stored at 4 C and developed after 20 45 and 85 days of exposure After developing the sections were stained with haematoxylin eosin methyl green pyronin and alkaline Congo red A cell was considered labelled if 4 or more silver grains could be counted over its nucleus A parallel series of not coated sections was prepared for P A S staining

RESULTS

Apart from group 0 no spontaneous deaths occurred among the recipient mice

The distribution and incidence of amyloid deposits among donor mice and the various groups of recipient mice corresponded fairly well to those of our earlier reported similar experiments (*Werdelin & Ranløv* 1966) In the *donor group* small amounts of amyloid were found in the spleens of 6 out of 12 mice while no other organs showed amyloid lesions Among the *recipients* no animals of the groups 0 and I showed amyloid lesions while one out of 5 recipients in group II showed minute amounts of amyloid deposits in the spleen In group III 4 out of 5 recipients contained amyloid in the spleen while in group IV all 5 recipients showed significant amounts of splenic amyloid As a general rule the incidence and severity of the amyloidosis increased with the length of time after the cell transfer As was the case in our earlier reported experiments the amyloid was found exclusively in the perifollicular zones of the spleens of the recipient mice

Autoradiographic investigations Most labelled cells in the *donor spleens* (Fig 2) *were found in the perifollicular regions The majority* of these were large cells with elongated chromatine weak nuclei containing one or two nucleoli A large proportion of these cells showed cytoplasmic pyroninophilia Other labelled cells were lymphocytes of various sizes and a few megakaryocytes Apart from the perifollicular zones the distribution of labelled cells throughout the splenic white and red pulps was rather uniform The labelling of the donor thymus was less pronounced most labelled cells being found in the thymic cortex In the donor liver relatively more Kupffer cells were found labelled than were the parenchymal cells



Figs 2 3

Fig 2 Aut radi graph of perif llicular region f d n r spleen (whit pulp at t right) labelled lymph id and reticular cell many f them h wing cyto plasmic pyr nin philia 45 days of exp sure Methyl green pyr nin (× 1400)

Fig 3 Aut radi graph of section f lung from recipient m u e kille l 5 minutes after cell transfer showing accumulati n f labelled d n r c ll in the capil laries Exp ure time 45 days Haemat xylin e sin (× 1400)

Figs 4

Fig 4 Aut radi graph f pleen f recipient m u e kille l 24 h u fter tran fer f hyperimmunize l d n r pleen cell labele l with H3 thy tine Labelled cell in the central area f the Malpighian c rpuscles (f ntral artery 5 days f exp ure Alkaline Gieg ref × 1400)

Fig 5 Aut radi graph f recipient spleen lay after cell tran f Labell l cel in the white pulp 45 days f exp ure M thyl green pyr 1400)

Fig 6 Aut radi graph f pleen fr m recipient m use killed after cell tran fer Labelle l cell are een in the p eif llicu ar regi n l in the re l pulp In a l liti n f lat l silver grains have appeare l t c l pla m f perif llicular reticul end thelial cell 45 days f exp M thyl green pyr nine (× 1400

Fig Aut radi graph f recipi nt pleen 4 lay after cell tran f l bell l c ll are seen in the perif llicular regi n an l in the red pulp l am ng n newl rmed amyl i l dep it hav appe re l t il m 4 f l xp u Methyl green pyr nin (1400

In the animals of the *recipient group 0* labelled cells were found exclusively in the capillaries of the lungs (Fig 3) No labelled cells could be recognized in the recipient spleens or livers The labelled cells were of the same type as those seen in the donor spleens many of them being pyroninophilic Quite often they could be seen in clusters forming small emboli In the recipient *group I* 24 hours after cell transfer practically all labelled cells had disappeared from the lungs and reappeared within the Malpighian corpuscles of the spleens In each section usually 8–20 labelled cells could be found distributed over a single Malpighian corpuscle with a tendency to accumulate in the more central parts near the central vessel (Fig 4) The labelling was confined to the nuclei At this stage only an occasional labelled cell could be recognized in the perifollicular region and labelled cells were never found in the splenic red pulp 2 days after cell transfer in the recipient *group II*, labelled cells were still confined to the splenic white pulp but now a tendency to accumulate in the perifollicular regions was apparent (Fig 5) The majority of labelled cells were of the large pale variety many being pyroninophilic At this stage labelling in the splenic red pulp was seldomly seen In the lungs the liver thymus intestines and kidneys extremely few labelled cells could be recognized In the recipient *group III*, killed 3 days after the spleen cell transfer the labelled cells seemed to have migrated further out now being found in the splenic red pulp and in the perifollicular zone of the admittedly now somewhat desorganized and shrunken Malpighian corpuscle (Fig 6) In 4 out of 5 animals of this group this localization coincided with the appearance in the same areas of small new formed amyloid deposits These findings were repeated in the recipient *group IV* killed 4 days after transfer In these animals significant amyloidosis was found in all the spleens The labelled cells were found in the same localizations as those of group III As was the case in the other recipient groups practically no labelling was found over other tissue sections neither was amyloid (Fig 7)

In addition to the well-defined nuclear labelling hitherto observed now isolated silver grains appeared over the perifollicular regions of the recipient spleens Most of these grains were apparently related to the cytoplasm of reticulo-endothelial cells probably representing phagocytized nuclear material derived from the labelled donor cells

DISCUSSION

The main observations of the experiment presented above are Firstly the major part of spleen cells from mice hyperimmunized with casein apparently migrate to the perifollicular regions of the spleen when injected intravenously into normal syngeneic recipients subsequently treated with nitrogen mustard This assumed migration is completed in the course of 2–3 days and seems to take place via the central vessel

of the Malpighian corpuscle Secondly the appearance of labelled cells in the perifollicular zone of the recipient spleen coincides with the appearance of amyloid deposits in the same region Finally the nitrogen mustard treatment necessary for the amyloid formation in the recipients seems to be accompanied by a significant destruction of the donor cells present in the recipient spleens

In the literature dealing with the fate and the organ distribution of transfused lymphoid cells conflicting opinions have been expressed These are mainly due to variations in genetical donor host relations variations in the type of donor cells employed and most often variations in the intervals between the transfusion and the killing of the recipient animals In general though most investigators agree that a major part of such transplanted cells after a certain length of time may be traced to the recipient spleens and to a lesser extent to the livers

In the present experiment animals dying within 15 minutes after the transfusion showed practically all of the injected labelled cells concentrated in the capillaries of the lungs This is in accordance with the findings of *Osogoe* (1950) and *Keohane & Metcalf* (1958) who found injected lymphoid cells accumulating in the capillaries of the lungs immediately after the injection after which they were gradually released into the blood stream In the present experiment 1 and 2 days after cell transfer labelled cells were found predominantly in the splenic white pulp after 24 hours with a tendency to accumulate around the central vessel This is in agreement with the observations of *Gowans* (1962) He found that between 3 and 12 hours after the transfusion of labelled lymphoid cells they could be found accumulated immediately around the central vessel in the white pulp of the recipient spleen This was true regardless of the genetic relation between donor and host In guinea pigs followed for 24 hours after the transfer of (allogeneic) isotopically labelled (Cr^{51}) lymphoid cells *Turk* (1962) found about one half of the activity remaining in the liver throughout the period while the activity in the spleen increased steadily from 1 to 6 per cent With a similar technique *Najarian & Feldman* (1965) studied the rate of isotope disappearance from the spleens of mice injected intravenously with H^3 labelled spleen cells from either syngeneic or allogeneic donor mice In syngeneic recipients the calculated half life of labelled cells was 82 hours in contrast to 40 hours in allogeneic recipients In an allogeneic donor host system *Hattler et al* (1964) found the majority of injected labelled spleen cells accumulating for 2 3 days in the recipient spleen In a rather indirect way the fate of injected allogeneic spleen cells was determined by *Haller* (1964) He found the mortality from "runting cut in half by removing the spleen of newborn mice shortly after the injection of allogeneic adult spleen cells His observations suggest that the injected spleen cells immediately settle out preferentially in the spleen of the new host from

which they initiate the lethal graft *versus* host reaction. From 4 to 21 days after the transfusion of allogeneic adult lymphoid cells to neonatal rats *Nowell & Defendi* (1964) found the injected cells proliferating more actively in the lymph nodes than in the spleen. In accordance with others (*Davies & Doak* 1960, *Howard, Michie & Simonsen* 1961, *Fox* 1962) their findings suggested the splenomegaly occurring later during the graft *versus* host reaction being mainly due to host cell proliferation. These observations suggest that the sojourn of injected lymphoid cells in the recipient spleen may be a temporary one, perhaps limited to a few days. However, that the strain combination may be decisive for the fate of injected parental cells must be emphasized. *Fox & Howard* (1963) found dividing cells of donor origin dominating the spleen after 3 weeks of graft *versus* host reaction in the strain combination C57Bl → (C57Bl × CBA)F1. From these observations it appears that conclusions regarding the fate of injected lymphoid cells into syngeneic recipients should not be drawn without criticism from experiments employing allogeneic or xenogeneic strain combinations.

Murray & Murray (1964) found H³-labelled thymus cells injected intravenously into syngeneic mice after a few hours sojourn in the capillaries of the lungs migrating primarily to the spleen and to some extent to the bone marrow and the lamina propria of the small intestine. In many instances these authors found evidence of transformation into other cell types ("blast-like") and they refused to rule out the possibility of a direct incorporation of labelled material into phagocytic cells.

That the origin of the "lymphoid" cells transferred to syngeneic recipients may be decisive for their destination within the recipient was illustrated in an interesting report by *Parrot et al.* (1966). These authors describe the preferential localization immediately around the central arteriole of the Malpighian corpuscle (the "thymus dependent area") of injected labelled thymus cells. Similarly labelled spleen cells also accumulate in these areas but, in addition, are distributed at the periphery of the splenic follicles. Many more spleen than thymus cells enter the lymphoid tissues and the spleen appears to be the primary target. Earlier experiments by *Diderholm & Fichtelius* (1958) with guinea pigs showed that transfused P³²-labelled thymocytes and lymph node lymphocytes both could be traced to the red pulp of the recipient spleens, mainly perifollicularly, though thymus cells seemingly could be recovered in the spleen to a greater extent.

Migration to the thymus of transfused lymphoid cells seems to be an uncommon finding. *Galton & Reed* (1966) injected H³-labelled spleen cells from syngeneic donor mice into normal recipients. After 3 days the vast majority of labelled cells was recognized within the Malpighian corpuscles of the recipient spleens and, in addition, a few labelled cells appeared in the thymus glands.

Even in syngeneic combinations difficulties may arise Recently *Celada* (1966) showed that mouse spleen cells in the donor animal primed to form antibody against human serum albumin failed to show the secondary response when challenged after transfer into intact mice of the same genotype This barrier which severely affected the donor cells capacity to implant and/or to function in the syngeneic system was found to be radiosensitive and to depend on the age of the recipient (being maximal after 2 months of age) In an allogeneic system *Hattler et al* (1964) found that donor cells presensitized with an indifferent antigen had survival times similar to normal donor cells

In the present material the relationship in time and topography between the appearance of labelled cells and amyloid deposits in the recipient spleens seems suggestive of a direct or indirect participation of these cells in the local production of the amyloid substance Evidence that amyloid is formed *in situ* by cells belonging to the reticuloendothelial system was first presented by *Teilum* (1956 1964) The present and our earlier experiments allow no conclusions to be drawn regarding the type of cells involved in this presumed local production of amyloid The majority of labelled cells found in our recipient spleens were apparently of the reticular variety but may not necessarily be identical with the large pale cells observed in the donor spleens as transformation into lymphoid type cells or *vice versa* under these conditions is known to occur (*Diderholm* 1961) Further less than 20 per cent of the nucleated donor cells were labelled under the conditions of the thymidine treatment and moreover the composition of this labelled cell population did not reflect that of the whole transfused spleen cell population

SUMMARY

Isotopically (H^3) labelled spleen cells derived from donors hyperimmunized with casein were transferred intravenously to normal syngeneic mice subsequently treated with nitrogen mustard The recipients were killed in series 0 1 2 3 and 4 days after the cell transfer From 2 to 4 days after transfer all of the recipient mice developed splenic amyloidosis Autoradiographic investigations revealed that the major part of the hyperimmunized donor spleen cells apparently migrated to the perifollicular regions of the recipient spleen This assumed migration was completed within 2–3 days and seemed to take place via the central vessel of the Malpighian corpuscle The appearance of labelled cells in the perifollicular zone of the recipient spleen coincided with the appearance in the same region of newly formed amyloid deposits

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SUBCUTANEOUS GROWTH OF EHRLICH'S ASCITES CARCINOMA AFTER SUBLETHAL WHOLE BODY IRRADIATION

By

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Received 17 xi 66

It has been shown previously that the early intraperitoneal growth of the Ehrlich ascites carcinoma (EAC) is not changed after prior treatment of the host with whole body irradiation in sublethal doses (*Thunold* 1966 a b) As this treatment was shown to abrogate the immune reactions the experiments indicate that this response is not of decisive importance to the intraperitoneal growth of EAC under these conditions Recent work however has shown that an increase in spleen weight and histological changes in the spleen and lymph nodes characteristic of antigenic stimulation occur during both intraperitoneal and subcutaneous growth of EAC (*Thunold* 1967) Contrary to intraperitoneal growth subcutaneous tumour growth seems to be influenced by these host reactions

The present work was planned to see if irradiation of the host 24 hours before transplantation might have any effect on the subcutaneous growth of EAC Changes in spleen weight and histology were examined 24 hours and 8 days after irradiation in non tumour bearing mice and 8 days after irradiation in tumour bearing mice

MATERIALS AND METHODS

Mice These were adult males and females of the closed colony kept at this Institute The mice were of similar age the males weighing between [illegible] and 2[illegible] g and the females weighing between 21 g and 23 g Two groups of [illegible] and 12 females and 3 control groups of [illegible] males and 8 females were set up

Tumour The Ehrlich ascites carcinoma used was [illegible] at [illegible] a female mouse of the closed colony

Irradiation Whole body irradiation (300 R) was g[illegible] previous [illegible] experiment (*Thunold* 1966 a)

Experimental [illegible]*dure*

Tumour growth and histology Whole tumour ascites (≈5 × 10[illegible] cells in 0 1 ml ascites) was g[illegible] subcutaneously on the back to groups of 1[illegible] and 1[illegible] female mice which had been irradiated or sham irradiated 2[illegible] hours previously The mice were killed b[illegible] cal d sloc[illegible]on on the 7th post transplantation day After removal

TABLE 1

The Mean Tumour Weight after Subcutaneous Injection of Ehrlich's Ascites Carcinoma in Control (EAC) and Previously Irradiated (EAC + IRR) Mice. The absolute and relative spleen weights are given in mice receiving irradiation only 24 hours and 8 days previously and in tumour bearing mice. Entries: means ± SD

Treatment	No. and sex of animals	Tumour wt (mg)	Absolute spleen wt (mg)	Relative spleen wt %
NIL	8 ♂	—	83 ± 12	0.39 ± 0.05
	8 ♀		82 ± 20	0.35 ± 0.09
IRR (24 hours)	8 ♂	—	40 ± 6	0.15 ± 0.02
	8 ♀		41 ± 4	0.18 ± 0.01
IRR (8 days)	8 ♂	—	66 ± 8	0.26 ± 0.04
	8 ♀		52 ± 17	0.23 ± 0.07
EAC	12 ♂	143 ± 45	250 ± 73	0.93 ± 0.31
	12 ♀	61 ± 42	305 ± 97	1.24 ± 0.35
EAC + IRR	12 ♂	155 ± 57	209 ± 26	0.76 ± 0.12
	12 ♀	149 ± 36	183 ± 17	0.77 ± 0.09

of the tumours through a dorsal skin incision both tumours and animals were weighed. Sections from formalin fixed tumours and from the tumour bed were stained with haematoxylin and eosin.

Spleen weight and histology. The spleen was removed in tumour bearing animals and the spleen weight recorded to the nearest 0.001 g. The relative spleen weight was calculated using the formula 100 × Wt. of spleen (g)/Wt. of animal (g). The relative spleen weight was also calculated in one untreated control group and in two

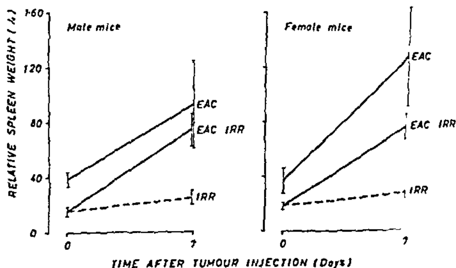


Fig. 1

The increase in relative spleen weight during growth of Ehrlich's ascites carcinoma (EAC) in normal and previously irradiated (IRR) male and female mice. The broken lines represent the change in relative spleen weight in mice without tumours between 24 hours and 8 days after irradiation.

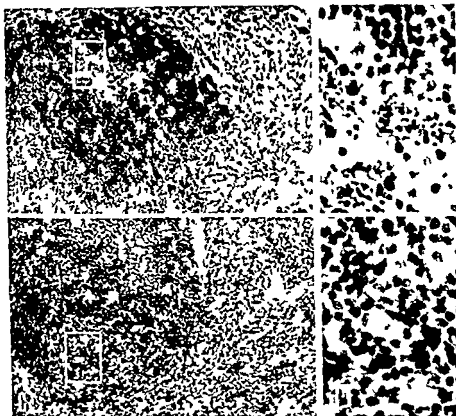


Fig 2

a Spleen from non tumour bearing mouse 24 hours after irradiation Oedema and large amounts of cellular debris and polymorphonuclear cells can be seen in the Malphigian body (left) There is no obvious changes in the red pulp Haematoxylin and eosin × 150

b Greater magnification (× 610) of the cellular debris seen in the inset in a

c Spleen from non tumour bearing mouse 8 days after irradiation There is less cellular debris in the Malphigian body (left) and that remaining is more closely packed Haematoxylin and eosin × 150

d Greater magnification (× 610) of the cellular debr s cen in the nset in c This finding indicates phagocytosis

groups of mice which had received irradiation onl 24 h r and 8 da previou ly respectively This was done as a control of the radiati n effe t p r se S ct on ere stained with haematox lin and eosin and methyl g een p on n a d examined microscop cally

RESULTS

Irradiated Non urro r Bearing Mice

The change in p c n weight after irradiation are shown in Table 1 and visualized in F 1 A significant decrease in relative spleen weight was found 24 h rs and 8 days after irradiation in both males (24 hrs



Fig. 3

a. Spleen from non-irradiated tumour-bearing mouse showing hyperplasia of the Malphigian body with activated germinal centre (left). The red pulp (right) is cellular and contains a number of erythrocytes. Haematoxylin and eosin × 150

b. Spleen from non-irradiated tumour-bearing mouse with relative absence of pyroninophilia in the Malphigian body (left). The red pulp contains a great number of pyroninophilic cells and some megakaryocytes (right). Methyl green pyronin × 150

$P < 0.001$; 8 days, $P < 0.05$) and females (24 hrs, $P < 0.001$; 8 days, $P < 0.01$). While the relative spleen weight showed no sex difference in control mice or in mice irradiated 8 days previously, it was greater in female than male mice 24 hours after irradiation ($P < 0.01$). No changes occurred in body weight after irradiation.

Histological examination of the spleens irradiated 24 hours previously revealed cellular debris and polymorphonuclear cells scattered in the Malpighian bodies (Figs. 2a, b). At 8 days after irradiation there was less cellular debris and that remaining was more closely packed (Figs. 2c, d). No obvious changes could be seen in the red pulp.

Non-Irradiated Mice Receiving LAC

The mean tumour weight (Table I) 7 days after transplantation was significantly greater in male than female mice ($P < 0.001$). Histologically the tumours showed small central areas of necrosis and haemorrhage. The connective tissue in the tumour bed showed ... infiltration of tumour cells and inflammatory cells, but no organized tumour tissue was seen.



Fig 5

a Spleen from irradiated tumour bearing mouse 8 days after irradiation Resting Malphigian body with some foci of cellular debris (left) and a less cellular red pulp Haematoxylin and eosin × 150

b Spleen from irradiated tumour bearing mouse 8 days after irradiation The Malphigian body (left) is relatively free from pyroninophilia and only a few pyroninophilic cells can be seen in the red pulp Methyl green pyronin × 150

c Greater magnification (× 610) of pyroninophilic cells seen in the red pulp in b

The increase in relative spleen weight after tumour growth was significant in both sexes ($P < 0.001$) The increase was more pronounced in female than male mice (Table 1 and Fig 1) Histological examination of the spleens revealed increased mitotic activity in the germinal centres and a cellular red pulp with erythropoietic activity m ny pyronino philic cells and a number of erythrocytes (Figs 3 a b) [illegible] and in the previous experiment (*Thunold* 1967)

Irradiated Mice Receiving EAC

The mean tumour weight was similar in m [illegible] group (Table 1) [illegible] ared to the control fe [illegible] in irradiated f [illegible] is increased ($P <$ 0 [illegible] ap- peared betwe [illegible] groups Histolo [illegible] these mice showed [illegible] milar to those [illegible] and the number of [illegible] ned to be the same

The incre [illegible] spleen weight during [illegible] growth in ir radiated mic [illegible] cant in both sex s ($P < 0.001$) This increase

was the same in the two sexes and it was remarkably similar in all animals as judged from the standard deviation of the means which were only 1/3 to 1/4 of the standard deviation in tumour bearing controls (Table 1 and Fig. 1).

The Malpighian bodies in these mice showed no mitotic activity and were less prominent than in the non irradiated tumour bearing controls. The red pulp was also distinctly less cellular than in the controls (Fig. 4 a) but some large pyroninophilic cells could be seen (Figs 4 b c).

Microscopy did not reveal any sex differences.

DISCUSSION

In this experiment the same growth pattern of subcutaneous transplanted LAC was observed in non irradiated mice as demonstrated previously (*Thunold* 1967) as the male mice showed larger tumours than the females. In contrast there was no sex difference in tumour growth in the irradiated animals. While the tumours in the two male groups were of equal size tumour growth in irradiated female mice was increased compared to the controls. There was slight and similar tumour cell infiltration of the surrounding tissue in all groups but no organized tumour tissue was seen in the tumour bed. This showed that tumour dissection could be considered satisfactory.

These group differences in tumour growth can be related to the differences in lymphoid reactions. In the non irradiated mice the females showed a higher degree of splenomegaly than the males while the splenic enlargement was similar in the two sexes in the irradiated group. The splenic enlargement in the non irradiated mice was accompanied by histological evidence of antigenic stimulation while the spleens of the irradiated tumour bearing mice showed less accumulation of large pyroninophilic cells and plasma cells in the red pulp and smaller Malpighian bodies with no distinct formation of germinal centers. The status of pyroninophilic cells as producers of antibody has been well established (*Fagraeus* 1948 *Coons et al* 1955) and evidence is accumulating for involvement of the germinal centers in the same process (*Gitlin et al* 1955 *Ortega & Mellors* 1957 *Mellors & Korngold* 1963). The histological findings thus indicate less immunological activity in the irradiated mice.

As depression of the immunological activity by sublethal irradiation measured by histological criteria is accompanied by increased tumour growth in female mice the present experiment supports the previous suggestion (*Thunold* 1967) that the immune response of the host is of consequence to the subcutaneous growth of LAC in this sex.

Decrease in immunological function of the mouse spleen after total body irradiation (400 R) has been studied by *Makinodan* (1962) who found an approximately 98 per cent decrease in agglutinin production

Using similar doses of X ray i e sublethal doses as in the present experiment *Argyris* (1963) found a similar decrease in immunological activity as measured by the ability of spleen cells to induce a graft versus host reaction The quoted data and the increased tumour growth in irradiated female mice in the present experiment may be attributed to cellular damage to the reticuloendothelial system caused by the previous irradiation A severe initial destruction of the germinal centres and loss of spleen weight was found 24 hours after irradiation in the present experiment i e at the time of tumour injection with substantial regeneration 8 days after exposure These findings are comparable with those published by others (*Henshaw* 1944 *Brecher et al* 1948 *Murray* 1948) on the effects of similar doses of X irradiation on the spleen and lymph nodes of mice

The previous (*Thunold* 1967) and the present experiment show histological changes in lymph nodes and spleen indicative of a host immune response during growth of EAC This homotransplantable tumour thus seems to contain antigenic components which are not shared by the host The acceptance and progressive growth of the tumour in spite of this antigenic difference appears to be dependant on two main vectors the size of the inoculum and the immunological defence of the host The dose used in these experiments is of a magnitude which seems to overwhelm the defence mechanism in the male sex while tumour growth in female mice is effected by a greater immune response in this sex

SUMMARY

Subcutaneous growth of the Ehrlich ascites carcinoma has been investigated in normal and previously irradiated mice and related to changes in spleen weight and histology

In non irradiated mice tumour growth was greater in the males than in the females The female mice showed a greater increase in spleen weight than the males and the spleens of both sexes showed histological evidence of immune response This suggests that tumour growth in the females has been inhibited by their greater immune resp[illegible] to the EAC

This is supported by the fact than when the im[illegible] d fence mechanism of th[illegible] mice is depressed by whole bod[illegible] [illegible]sured by histological criteria no sex difference is appa[illegible] [illegible] size or host re[illegible]tion and tumour growt[illegible] [illegible] significantly gr[illegible] han in non irradiated fe[illegible]

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PRIMARY GERM CELL TUMOR OF THE ANTERIOR MEDIASTINUM WITH FEATURES OF ENDODERMAL SINUS TUMOR

(*Mesoblastoma Vitellinum*)

By

INGER TEILMANN[1] HARRY KASSIS and GIUSEPPE PIETRA[3]

Received 16 xii 66

The anterior mediastinum and the pineal gland share the curious property of being the site of predilection for the development of the extragonadal germ cell tumors Teratomas embryonal carcinomas choriocpitheliomas and seminomas have been described in these locations (*Friedman* (5) *Kountz et al* (7) *Oberman et al* (10) *O Gara et al* (11) *Inada & Nakano* (8) *Lattes* (9)) In contrast to the adult testes however most of the germ cell tumors in extragonadal location are benign The reason for this difference and for the location of primordial germ cells in these organs is unknown The study of this interesting group of neoplasms is impaired by their rarity and justifies the reporting of single cases In the ovary and in the testis *Teilum* (15 16) described germ cell neoplasms characterized by a mixture of undifferentiated neoplastic mesoblastic cells and irregular spaces closely resembling the endodermal sinuses of Duval characteri[illegible]tic of the rat s placenta (*Duval* (4)) The author considered this type of neoplasm as an extraembryonic membrane tumor morpho[illegible] c[illegible]lly dis[illegible]inct from the extremely [illegible] group of the embryon[illegible] c[illegible] [illegible] [illegible]as The follow ing report of a cas[illegible] of anterior mediastinal pr[illegible] g[illegible]m cell tumor with endoderm[illegible] [illegible] features is the first [illegible] d[illegible] u[illegible]entatio[illegible] of such a tum[illegible] [illegible] anterior mediastinum [illegible] [illegible] of extrag[illegible] dal origin We [illegible] [illegible] more cases will be r[illegible]ized [illegible] the [illegible] racteristic fe[illegible]es of this neoplasm will become more [illegible] [illegible]own

[1] Research [illegible] Pathology Present address The Univ[illegible] [illegible]nstitute of Pathological A[illegible]t my Co[illegible] agen Denmark
[2] Resident [illegible] [illegible]thology
[3] Patholog[illegible]

CASE REPORT

A thirty three year old Negro man was admitted to Michael Reese Hospital and Medical Center because of fatigue weight loss mild substernal pain and cough productive of black stained sputum His symptoms had initiated ten months earlier and had become progressively more severe during the past four months The family and personal history was non-contributory On admission physical examination disclosed no significant findings other than moderately enlarged left cervical and supraclavicular lymph nodes Laboratory data were within normal range A chest x ray and tomogram revealed a large anterior mediastinal mass which displaced the trachea posterior The clinical impression was that of a malignant lymphoma or a thymoma although the possibility of a teratoma could not be entirely ruled out Biopsy of the supraclavicular lymph nodes revealed only non specific lymphoid hyperplasia

On the seventh hospital day mediastinoscopy revealed a large tumor mass which occupied the anterior mediastinum surrounded the large vessels and extended into the right pleural space The mass was of deep gray purple color and was extensively necrotic It could be only partially resected in view of its relationships with the superior vena cava and the innominate vein

Following surgery the patient made an uneventful recovery although cytological examination of the sputum revealed the presence of malignant cells indicating extension of the tumor to the tracheo bronchial wall He was discharged two weeks later and followed in the out patient department He received 12,210 r to the anterior mediastinum in twenty seven treatments over a period of thirty eight days He appeared to tolerate the treatment well however two months following his first admission he noticed bilateral subcutaneous masses in the anterior chest wall which were considered as metastases For these additional 5000 r were administered by means of Co 60 radiation In spite of therapy he became progressively weaker had repeated hemoptysis and became markedly anemic The second admission was four months following discovery of the tumor At this time there were left supraclavicular and posterior cervical lymphadenopathy and left pleural effusion A test for urinary gonadotropin was negative The patient received Cytoxan Nitrogen mustard and Tetracycline intrapleurally Despite all therapeutic efforts he declined rapidly and expired in marked respiratory distress five months after the initial diagnosis had been made

Pathology

The surgically removed tumor consisted of a partially encapsulated gray purple mass which measured 11×8×6 cm and weighed *208* gm On section it was gray purple in color soft centrally necrotic and hemorrhagic

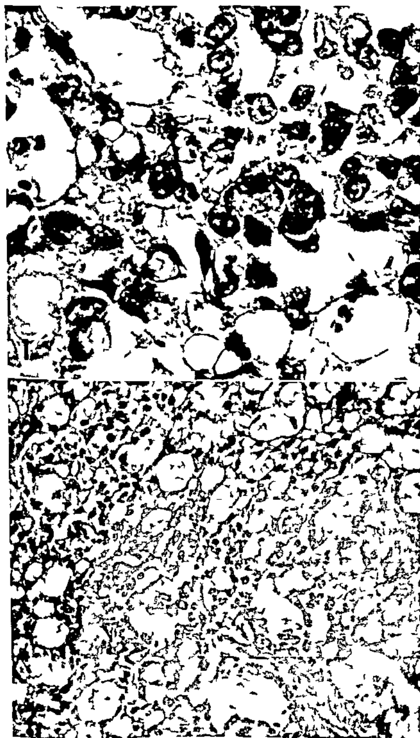
Microscopically the tumor was composed of several cell types The main portion was formed by solid masses of large cells with clear cytoplasm and round to pleomorphic nuclei with delicate chromatin and prominent nucleoli (Fig 1) Intermingled were *areas of stellate* cells connected by delicate projections and forming a loose network with wide *meshes* (*Fig 2*) Imperceptibly merging with these areas of undifferentiated cells were irregular spaces lined by low cuboidal or

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Figs 1 2

Fig 1 The tumor is composed of solid nests of large undifferentiated cells with clear cytoplasm. Some of the cells have large cytoplasmic [illegible] Note the cystic spaces lined by flattened cells. (*H E × 613*)

Fig 2 Areas showing clumps of markedly vacuolated cells forming delicate cobweb like structures with developed microcysts (*H E ×* [illegible]



flattened cells with large round or indented hyperchromatic nuclei and scanty cytoplasm The cavities appeared to communicate with each other forming an intricate system of irregular channels in which papillary structures projected (Fig 3) The papillae were covered by a single layer of epithelial like cells supported by delicate connective tissue containing thin walled blood vessels On cross section these papillary structures appeared as peculiar glomerularlike formations with a single capillary in the center covered by a mantle of cylindric cells of epithelial character (Fig 4) These structures (Fig 5) have been interpreted as invaginated yolk sac endoderm accompanying the vessels of the extra embryonic mesenchyme (15 16) In the cytoplasm of both the flattened cells and in the epithelial like cells there were eosinophilic strongly PAS positive droplets In addition extracellular accumulations of such hyaline globules were often present (Fig 5)

At the periphery the tumor mass was partially surrounded by dense collagen in which residual thymic tissue was present (Fig 6) Within the tumor mass were small lymphoid foci possibly representing residual lymphatic tissue of the thymus

As the cells of the sputum appeared quite unusual a description of these may be of interest The cells appeared mostly single or in pairs Some of the cells had large cytoplasmic vacuoles Large nuclei which appeared round or oval with distinct nuclear membranes and finely dispersed chromatin Some were hyperchromatic lobulated and irregular in outline with chromatin clumping (Fig 7) The nucleoli varied in size and shape

At autopsy the original surgical site was entirely obliterated by tumor regrowth and the anterior mediastinum beneath the sternum was occupied by a large yellowish gray tumor mass with multiple areas of hemorrhagic necrosis and an outer layer of intense reddish brown soft tumor tissue extending to the entire left parietal and visceral pleura and lung The thymus gland was not recognized as such Hemorrhagic and sero fibrinous fluid filled most of the left pleural space with subsequent compression of the lung On the right side the pleural lining and cavities were free of tumor however there was presence of confluent bronchopneumonia and multiple thromboemboli within the peripheral vessels of the lungs

In addition to local extension the tumor had metastasized widely throughout the body Almost all of the thoracic lymph nodes were involved bilaterally including the mediastinal hilar and peribronchial lymph nodes as well as the supraclavicular and para aortic lymph

Figs 3–4

Fig 3 Systems of irregular channels lined by low cuboidal epithelium Note the papillary formations with a blood vessel in their centre In cross section such structures vaguely resemble immature renal glomeruli (H E × 90)

Fig 4 Endodermal sinus structure with perivascular mantling of cells (H E × 24[illegible])





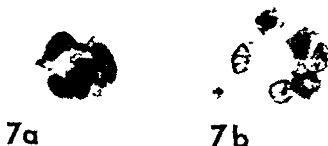


Fig 7

(a and b) Tumor cells in the sputum Note in *a* the large oval shaped hyperchromatic nuclei and the scanty cytoplasms In *b* the cell on the left has a large cytoplasmic vacuole the remaining cells have round or lobulated nuclei clumping of the chromatin and nucleoli of varying size (Papanicolaou × 5__)

nodes Metastases were present in the liver and in the skin of the left anterior chest wall Careful attention was given to the examination of the testes No tumor tissue was seen grossly or microscopically in any of the multiple sections examined by the method of serial blocks Further, there were no areas of scarring in the testes A striking feature was the widespread atrophy of the germinal epithelium and hyaline thickening of the tubular basement membrane The tubules were lined by Sertoli cells and few scattered spermatogonia The interstitial cells were well preserved and did not appear increased in number (Fig 8)

Microscopic examination of the tumor revealed essentially the same characteristic features seen in the surgical specimen (Fig 9) In addition many of the metastatic masses showed a more undifferentiated pattern In some of the sections of the recurrent mediastinal tumor there were large cells with strongly eosinophilic cytoplasm and large hyperchromatic nuclei resembling syncytiotrophoblastic cells

DISCUSSION

As illustrated in the Figs 1 through 5 and Fig 9 the anterior mediastinal neoplasm reflects the histologic features of the endodermal sinus tumor as described by *Teilum* (15 16) in the ovary and testis These

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Figs 5–6

Fig 5 Area of the mediastinal tumor showing the characteristic endoderm sinus structure representing yolk sac endoderm that expands and dissects around the vessels of the extraembryonic mesenchyme The structure is surrounded by a loose vacuolated network of stellate mesodermal cells and small cystic spaces showing focal accumulations of PAS positive hyaline globules H E × 200)

Fig 6 Well preserved thymic tissue at the periphery of the anterior mediastinal tumor Note the Hassal's corpuscle (*H E × 240*)



Fig 8

Representative section of the testis The tubules are lined by Sertoli cells and isolated spermatogonia Note the *marked thickening of the tubular basement membranes* (H D × 60)



Fig 9

Pulmonary metastasis The tumor although generally more undifferentiated in the metastases reveals focally the typical endodermal sinus pattern (H D × 150)

are (a) areas formed by stellate mesodermal cells forming a loose vacuolated network delineating irregular spaces lined by flattened endothelial like cells and often containing foci of active hemopoiesis (b) endodermal sinus structures characterized by perivascular arrangement of cylindric epithelium which in cross section vaguely resemble immature glomeruli They are surrounded by cystic spaces lined by a single layer of flattened mesothelial like cells with prominent nuclei In longitudinal section they appear as a complete labyrinth resembling the endodermal sinuses of the rodent placenta (c) compact masses of undifferentiated cells similar to the undifferentiated cells of the developing embryo and (d) cystic structures lined either by flat mesothelial like cells or by low cuboidal epithelial like cells These cysts are considered analogue to the yolk sac of the early embryo

The proportion of the various patterns vary considerably not only in different tumors but also in different areas of the same tumor In the present case the features mentioned in (d) were not seen However there was a striking resemblance to the ovarian and testicular tumors described by *Teilum* (15 16) as endodermal sinus tumor with the testicular neoplasms of infant testis reported by *Huntington et al* (6) and with the sacrococcygeal teratoma of *Raghunatha et al* (12) also considered examples of endodermal sinus tumors

In Teilum s opinion this tumor represents the differentiation of entirely undifferentiated neoplastic embryonal cells (15 16) into extra embryonic membrane structures such as the mesoblast and yolk sac endoderm Teilum s interpretation of the histogenesis of these neoplasms was based on comparative studies which among other things showed a striking resemblance of the perivascular sinusoid structures in the tumor with the embryologically well defined endodermal sinus structures which are prominent in the rat placenta In both instances these formations represent diverticula of yolk sac endoderm that expand and dissect around the vessels of the extraembryonic mesenchyme While this comparison is valid not only in regard to the general appearance in cross and longitudinal sections but also to the type of lining cells it would not imply a specific capacity of the tumor to reproduce phases of placentation in the rodent (17)

The ovarian tumors show a far more uniform histologic pattern than their testicular counterparts in adults which usually reveal great variations of immature embryonic tissues The term embryonal carcinoma has been a subject of criticism since it lacks clarity and leads often to confusion by its ambivalent use For practical use the term may be retained to designate these complex germ cell tumors of the adult testis which comprise a fairly homogenous *clinical* group However it is not sufficient basis for histologic characterization and recognition of the ovarian counterparts and similar tumors in the infant testis or extragonadal positions (16)

In our case the pattern of endodermal sinus tumor was striking in

the resected tumor but only found locally in the recurrent tumor and its metastases (Fig. 9). Clinically the neoplasm as the embryonal carcinoma of the adult testis affect the same age group and is resistant to radiation or chemotherapy. In contrast to the behaviour of this type of neoplasm in children all cases of endodermal sinus tumor in the adult have a malignant course regardless of treatment.

It is well known that gonadal malignant teratomas, embryonal carcinomas and choriocarcinomas may metastasize solely to the mediastinum. In this case, however, serial blocks of the testis failed to show any tumor or scar. In addition thymic tissue was identified at the periphery of the neoplastic mass (Fig. 6). It is then reasonable to assume that this tumor arose in the thymus gland or in its proximity. There is no agreement as to the factors which lead to the development of gonadal and extragonadal teratomas and embryonal carcinomas. *Willis* (18), *Collins & Pugh* (2) deny their origin from germ cells and believe that they arise from foci of plastic pluripotential embryonic tissue which have escaped from the influence of the primary organizer during fetal life. Most authors, however, believe in their origin from germ cells through different stages of development (*Friedman* (5)), *Dixon & Moore* (3), *Teilum* (15, 16), *Bhargava & Reddy* (1). *Schlumberger's* (13) interpretation that the mediastinal germ cell neoplasms arise from the third branchial pouch, the anlage of the thymus, seems unlikely. It is now generally accepted that the germ cell neoplasms in this region are derived from germ cells which have been misplaced during ontogeny and that during their passage from the yolk sac endoderm they have travelled through the retroperitoneum to the mediastinum instead of the gonadal ridges (*Teilum* (17)). Of interest was the presence of a marked degree of atrophy of the germinal epithelium and the thickening of the basement membranes of the tubules of the testis. No explanation is readily available for this process. It may have been the result of inanition and chemotherapy, although the latter appears unlikely in view of the findings of *Oberman & Libcke* (10). Three patients in their series of mediastinal malignant germ cell tumors had similar testicular findings. These authors do not state which one of the patients of their series had testicular tubular atrophy but only one was treated with nitrogen mustard, the others received only x-ray therapy or were surgically treated. No explanation has been offered to clarify the striking preference of the malignant germinal tumor for the male while the incidence of the benign counterpart is approximately the same in both sexes. Similar contrasting ratios of malignant and benign teratomatous tumors in the two sexes are seen also in the gonadal tumors. Here the suggestion has been presented ((7) p. 53 and (14) p. 226) that the environment in the female gonads predisposes germ cell neoplasms to differentiation, whereas the male environment fosters undifferentiated growth. This hypothesis could be extended to extragonadal germ cell tumors. It is conceivable that in the female the same

genetic material has greater potency to differentiation possibly because of a more suitable endocrine environment In this respect it is of interest that the endodermal sinus tumors—which in *Teilum* s (15 16) view are extra embryonic and in fact endodermal derivatives from germ cells—are rare in the adult testis but common in the testis of the young child and in the ovary

SUMMARY

The first case of mediastinal germ cell tumor with endodermal sinus features in a thirty three year old Negro male is reported The neoplasm exhibited the predominant pattern of communicating cavities lined by cuboidal or flattened cells and papillary structures as described in the ovary originally by *Teilum* The tumor was rapidly fatal and metastasized widely

The specific histological features of endodermal sinus tumors in this and other regions will enable their proper identification and distinction from other types of neoplasm

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THE RELATIVE BIOLOGICAL EFFECTIVENESS OF HIGH ENERGY RADIATION ON THE MITOTIC ACTIVITY IN NORMAL HEPATIC REGENERATIVE TISSUE

By

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The relative biological effectiveness (RBE) of some radiation r compared to some standard radiation s is defined by D_s/D_r where D_s and D_r are respectively the absorbed doses of the two beams in the region of interest that produce an identical biological response As RBE can also be used the ratio of the radiation effects which can be produced by the same absorbed dose In this work the latter definition is used

The RBE of different types of radiation has usually been studied by indirect methods for example by total body irradiation of animals and observation of the 50 per cent mortality ($LD_{50/30d}$ = lethal $dose_{50/30d}$ = the dose required to cause 50 per cent deaths of the irradiated animals within a 30 day period) as *Paterson et al* did They irradiated mice with 300 kV roentgen rays and with X rays from a 4 MeV linear accelerator LD_{50} was achieved with 560 rad and the linear accelerator with 744 rad which gave an RBE of 0 743 ± 0 03 for the latter Using irradiation of the thymus and the spleen and observing the weight loss the RBE was 0 858 ± 0 14 and the RBE for drosophila was 0 82 Another method of RBE determination is based on tissue culture radiation By irradiating for an observation period of 24 hours tissue cultured fibroblasts of chick heart and HeLa cells of pavement epithelium carcinoma of portio uteri

Gartner (1955 1958) among others determined the RBE for electrons (15 MeV) and compared the effect with that of 180 kV X rays He noted the number of mitoses mitotic phases injured mitoses and compared the effect of different types of radiation by using doses from 250 R to 2000 R at rates of 40 R/min and 1000 R/min He found that the RBE values increased with the dose rate and total dose The RBE with electrons was close to 1 when the dose rate was 1000 R/min and the total dose exceeded 500 R

Gartner & Zoepprit found that the number of mitoses was reduced

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and that of pathological mitoses increased with an increasing total dose of beta radiation emitted by Sr[90] *Bauer & Gärtner (1962) found that* continued irradiation heightens the biological effects

Total arrest of tumour growth afer X ray therapy and cobalt therapy was studied by *Linden* (1964) who determined the maximum dose that needed to destroy Walker cancer of the rat X ray radiation destroyed it with 2500 R and cobalt with 3500 R The RBE value obtained from these results for the gamma radiation of cobalt was 0 72 ± 0 1

The relative biological effect has been studied comparatively little *in vivo* by quantitative methods in the high energy range The present study is an attempt to explain this question *in vivo* by using different qualities of radiation and by giving one dose and comparing its effect on mitotic counts phases and injured mitoses

MATERIAL AND METHODS

Three month old white male rats (Sprague Dawley) were used for the experiment *Six groups each of 10 animals weighing 195 240 g were kept in the same cage at* +20 C To produce regeneration of the liver 70 per cent hepatectomy was performed under ether anesthesia by removing the median and left lobes according to the method of *Higgins & Andersson* (1931)

The time chosen for irradiations of varying energy was 48 hour after hepatectomy This is when mitotic activity is at its highest after hepatectomy according to *Lahtiharju* and it is thus possible to discern the effect of irradiation on the mitoses more accurately The liver was then given a dose of 300 rad at a rate of 40 rad/min at maximum dosage as previously employed in some methods of irradiation by (Irtner (1955 1958) The 300 rad dose was chosen because it is the conventional single dose in radiotherapy Radiation was given to the animals in the different groups by 200 kVp X rays (HVL 1 6 mm Cu) cobalt 32 MeVp betatron X rays and both 35 and 20 MeV betatron electrons One group of 10 rats served as the control group The irradiation time was at 7 p m and the technical methods were as follows

The rat was surrounded by water equivalent material which consists of paraffin beeswax and talc so that density is 1 g/cm³ Irradiation to keep place in 8 × 8 sq cm field so that the rest of the liver was situated at the point of maximum dosage (Fig 1) *During irradiation the dose being delivered was monitored by and* [illegible]

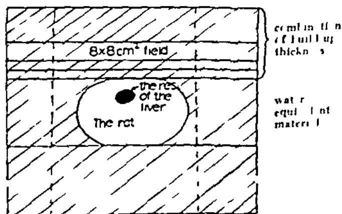


Fig 1

The irradiation of the liver of the rat by different radiations.

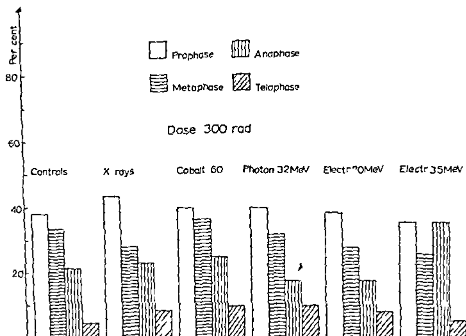


Fig 2
The mitoti phases after 12 hours by using different radiations

tion chamber which was checked against Max Planck Institute calibration according to *Fretschko et al* (1961) It can thus be assumed that the only differences involved in the irradiation were in the physical characteristics of the five beams and that it must be these therefore which are responsible for any biological differences observed For the conversion of roentgens into rads the stopping power and density effect according to the *National Bureau* (1961 1964) and *Johns* (1961) were taken into account

The animals were decapitated 12 hours after irradiation Tissue specimens were taken immediately from the liver fixed in Bouin s solution and stained with hemalumeosin Mitoses were counted per 100 fields with a binocular microscope magnification $10 \times 100 \times 1.5$ over an area of 0 785 sq mm

The following points were observed mitotic counts mitotic phases pathol gical and multipolar mitoses

RESULTS

The mitotic counts were highest in the controls 10 mitoses per 100 fields (Table 1) X rays gave the lowest and 20 MeV electrons the highest mitotic index After subtracting the mitotic indexes for differ en irradiation groups from the mitotic index for the control group the RBE was calculated by dividing the remainde s of each irradiation group by the remainder of the X ray mitoti inde (10 ?–4 0 = 6 2) The RPF values for the different irradiation groups ar shown in Table 1

There were no significant differences in the occurrence of different mitotic phases after exposure to different sources of radiation (Fig 2)

TABLE 1

Mitotic Index, Pathological and Multipolar Mitoses and RBE Value of the Liver after Hepatectomy [illegible] Hours after Irradiation with X rays, Cobalt 60, Electrons and Betatron Photons

	Control	X rays	Cobalt 60	32 MeV Photon	20 MeV Electron	35 MeV Electron
			Each Group Comprises 10 Animals			
Mitotic Index Mean	10 2 ± 0 9	4 0 ± 0 4	4 6 ± 0 4	5 2 ± 0 9	7 0 ± 0 4	6 2 ± 0 9
Mitotic Loss Mean		6 2 ± 1 2	5 6 ± 1 2	5 0 ± 1 7	3 2 ± 1 2	4 0 ± 1 8
RBE		1	0 90 ± 0 6	0 80 ± 0 31	0 52 ± 0 22	0 64 ± 0 31
Pathological Mitoses per cent	5 0	35 0	36 0	27 0	46 0	35 0
Multipolar Mitoses per cent	0 1	–	0 1	0 1	0 5	0 1

Mitotic count mean

Prophases were most numerous in all the groups except in the 35 MeV electron group where prophase and anaphase phases were the same (35 per cent)

Pathological mitoses produced by irradiation were found in equal numbers after X rays cobalt and 35 MeV electrons some 35 per cent the maximum 46 per cent was noted after 20 MeV electrons and the minimum 27 per cent after photon irradiation Multipolar mitoses were also most frequent after 20 MeV electron irradiation (Table 1)

DISCUSSION

The time chosen for taking the biopsy specimen was 12 hours after irradiation since according to *Gärtner et al* the rise in the mitotic index the secondary effect is at its highest 9–12 hours after irradiation of 250–2000 R in the tissue culture

X ray irradiation had the most pronounced effect on mitotic counts (4 0 ± 0 4 mitoses per 100 fields Table 1) and 20 MeV electrons the least pronounced (7 0 ± 0 4) The value for cobalt was 4 6 ± 0 4 When the RBE value of X rays was 1 that for cobalt was 0 9 ± 0 26 which exceeds the value reported by *Linden* (1964) in experiments with Walker cancer This suggests that the RBE in tumour is different from that in normal growing tissue The RBE values of photons were 0 80 ± 0 31 of 20 MeV electrons 0 52 ± 0 22 and of 35 MeV electrons 0 64 ± 0 31 The results for the different types of irradiation differ from one another very rarely Thus the RBE value cannot be regarded as reliable for different kinds of irradiation

Definite radiation damage was seen in dividing cells after the different types of radiation It was relatively most marked after 20 MeV electron radiation and the percentage of pathological mitoses was 46 Pathological mitoses were most numerous after electron radiation and fewest after photon radiation With all the sources of radiation pathological mitoses increased significantly compared with the controls They were most frequent in the anaphase and telophase—about 80 per cent—as *Friedrich et al* (1964) also noted when they irradiated the root tip cells of Allium cepa with 15 MeV electrons

SUMMARY

The regeneration of the liver of rats was produced by performing 70 per cent hepatectomy After two days the rest of the liver was irradiated with a dose of 300 rad at the rate of 40 rad/min Radiation was given by 200 kV X rays cobalt 32 MeV X rays of betatron photons and both 20 and 25 MeV betatron electrons Tissue specimens were taken 12 hours later from the liver and mitoses were counted per 100 fields X rays gave the lowest and 20 MeV betatron electrons the highest mitotic index The pathological mitoses were most frequent after electron

radiation fewest after cobalt irradiation On the basis of the mitotic frequencies the RBE (relative biological effectiveness) of the different types of radiation was calculated taking the RBE for 200 kV X rays is 1 The RBE value for cobalt radiation was 0 9 ± 0 26 and for the fast electrons and photons the values were smaller

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THE EFFECT OF HIGH ENERGY RADIATION ON THE MITOTIC ACTIVITY AND RELATIVE BIOLOGICAL EFFECTIVENESS IN WALKER TUMOURS

By

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Received 25 ii 67

The effect of different types of radiation has been studied by observing the $LD_{50/30d}$ (Lethal dose 50 per cent deaths in 30 days) *Paterson et al* (1957) in total body irradiation of mice and by irradiating tissue cultured normal cells or malignant cells and observing the mitotic activity and determining the relative biological effectiveness of electrons *Gartner* (1958) and *Friedrich et al* (1964)

The relative biological effectiveness (RBE) can be expressed either as a ratio of doses which produce a given effect or as a ratio of effects following equal doses In this work the latter definition is used In an early work by *Voutilainen et al* the same question was studied in vivo by irradiating the rest of the liver 2 days after 70 per cent hepatectomy when mitotic activity is at its highest X rays gave the lowest and betatron electrons of 20 MeV the highest mitotic index The RBE for X rays of 200 kV was 1 and for cobalt radiation 0 9±0 26 For the fast electrons and photons the values were smaller

The tumour growth after X ray and cobalt therapy was studied by *Linden* (1964) in Walker cancer The RBE value obtained for the gamma radiation of cobalt was 0 72±0 1

The present study is an attempt to explain the effect of high energy radiation on the mitotic activity and RBE value in Walker cancer

MATERIAL AND METHODS

Three month old white rats were used for the experiment These CB tock rats with Walker cancer were supplied by the Chester Beatty Research Institute in London Groups of 10 animals weighing 250–350 g were kept in the same cage at 20 C The tumour was implanted under the skin of the interscapular region After about 10 days when the tumour was spherical and about 1 5 cm in diameter the irradiation was given The rat was surrounded by tissue equivalent material dimensions 20 × 20 × 20 cu cm consisting of paraffin beeswax and talc and making up a density of 1 g cu cm The paraffin beeswax ration was 2 1 The top of the tumour

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(a depth of 0.5 cm under the skin) was always situated at the point of measurement. The tumour absorbed dose was a constant 300 rads. Tissue specimens were taken after the irradiation in the region where the calculated thickness was 0.5 cm.

The radiation sources and the exposure conditions were as follows:

200 kVp X Rays. The standard beam was from a Siemens constant potential X ray machine "Stabilipan 250" operated at 18 ma with a total filtration of 1.0 mm Cu. HVL of 1.6 mm Cu. The distance from the X ray target to the phantom surface was 50 cm giving a field size of 8 × 8 sq cm and the absorbed dose rate was 40 rad/min at a depth of 1 cm. The absorbed dose ratio of maximum to minimum was about 1.03.

^{60}Co γ Rays. A Siemens "Gammatron – 1" teletherapy unit. The activity of the source was approximately 2000 curies. The distance from the source to the phantom surface was 91 cm giving a field size of 8 × 8 sq cm and a peak absorbed dose rate of 40 rads/min at a depth of 0.5 cm. The absorbed dose ratio of maximum to minimum was about 1.07.

32 MeVp Photons. From a Brown Boveris "Asklepitron 35" betatron operated at 32 MeV. The distance from the target to the phantom surface was 100 cm, the field size was 8 × 8 sq cm and the peak absorbed dose rate approximately 40 rads/min at a depth of 5.5 cm. The absorbed dose ratio of maximum to minimum was about 1.

20 MeV Electrons. The electrons were accelerated by the same betatron as above. The distance to the phantom surface was 110 cm giving a field size of 8 × 8 sq cm and a peak absorbed dose rate of approximately 40 rads/min at a depth of 1.5 cm. The absorbed dose ratio of maximum to minimum was about 1.

35 MeV Electrons. As above.

Calibration for all five radiations was conducted with a 250 R Victoreen chamber, *Sinclair et al.* (1958) and Baldwin Farmer sub-standard dosimeter MK 2. Both were checked against Max Planck Institute calibration according to *Kretschko et al.* (1961). The Fricke ferrous sulphate dosimeter was used (*Schulek et al.* 1962). The dose measurements and high energy electrons were compared at the betatron in Helsinki and Umeå, Sweden, in December 1964 (*Hettinger* 1965). The dose values in Helsinki and Umeå were in good agreement with a maximum deviation of 3 per cent according to *Pettersson et al.* (1965). The comparison was performed with Fricke also against Vienna, Austria, when the difference was less than 1 per cent (*Vogl et al.* 1965). The conversion of roentgens into rads was calculated according to the National Bureau (1961 and 1964). The irradiation time was 7.5 min.

The animals were decapitated 12 hours after irradiation. Tissue specimens were taken from the tumour 0.5 cm under the skin, fixed in Bouin's solution and stained with hematoxylin-eosin. Mitoses were counted per 100 fields with a binocular microscope magnification 10 × 100 × 1.5 over an area of 0.785 sq mm.

RESULTS

The mitotic counts of the controls and the different radiation groups are given in Table 1. They were highest in the control group (39.2 ± 2.7). In the irradiation material the X ray group gave the lowest (9.9 ± 0.9) and the 35 MeV betatron electrons group the highest mitotic index (26.7 ± 2.9). The other mitotic counts were: cobalt 15.1 ± 2.1, photons 21.4 ± 1.9 and 20 MeV betatron electrons 25.1 ± 2.0.

After subtracting the mitotic indexes for the different irradiation groups from the mitotic index for the control group the RBE value was calculated by dividing the remainders of each irradiation group by the remainder of the X ray mitotic index (39.2 − 9.9 = 29.3). The RBE values for the different irradiation groups are shown in Table 1. The RBE values ranged from 1 to 0.38.

The pathological mitoses produced by irradiation were lowest in the cobalt group (15 per cent) and highest in the 32 MeV photon group (62 per cent) (Table 1).

TABLE 1

Mitotic Count Loss and RBE of Different Radiations in Malignant Tissue

	Control	X Rays	Cobalt 60	32 MeV Photon	20 MeV Electron	35 MeV Electron
Mitotic Count Mean ± S D	39 2 ± 2 7	9 9 ± 0 9	15 4 ± 2 1	21 4 ± 1 9	25 1 ± 2 0	26 7 ± 2 9
Mitotic Loss Mean ± S D		29 4 ± 3 6	23 9 ± 4 8	17 9 ± 4 6	14 1 ± 4 7	11 0 ± 5 6
RBE		1	0 81 ± 0 19	0 61 ± 0 17	0 48 ± 0 17	0 38 ± 0 20
Pathol Mitoses per cent	8 0	40 0	15 0	62 0	22 0	19 0

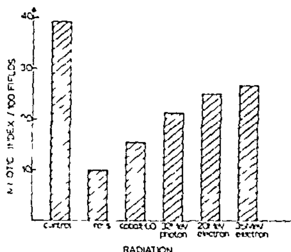


Fig. 1
Mitotic activity of controls and the other groups after different radiations in Walker-cancer

DISCUSSION

To compare the mitotic indexes (Fig. 1) obtained in the present work with the results of irradiation of normal degenerative tissue the same point of time *i.e.* 12 hours after irradiation was chosen for taking the samples. This time is suitable for the mitotic index for the control index was fairly high in this tumour tissue (39.2±2.7). The mitotic indexes of the irradiated groups were lower than the control index and higher than in an earlier study but the fluctuations were similar in that mitoses were fewest in number (9.9±0.9) after X rays and most numerous after 35 MeV electrons and the result was almost the same (25.1±2.0) with 20 MeV electrons.

The effect of different types of irradiation on mitotic activity differed (Fig. 1). It can be said that the mitotic index grows with the energy of the radiation both with roentgen gamma rays and with the electrons used. Secondly it must be noted that pathological mitoses are most numerous in the X ray and 32 MeV photons groups and significantly fewer in the groups given cobalt and both electrons (Table 1).

It is obvious that different types of irradiation at a certain time in this case within 12 hours of the irradiation exert differing effects. One such effect is the changing of the mitotic cycle: cell division is lengthened as *Albert et al.* (1960) established.

It is possible that the lengthening of the cell division is manifested differently in the irradiation groups and the result is a change in the mitotic cycle which is enough to produce great differences in rapidly growing tissue. The mitotic figures after roentgen and cobalt irradiation may correspond with reality as late as 12 hours after the irradiation whereas the effect of higher energy photons and electrons is

manifested as a prolongation of the mitotic cycle and thus as an ostensible increase in the mitotic figures

The pathologic mitoses established in all the irradiation groups are typical irradiation effects and their variations in the different groups are considerably greater than in the corresponding groups in normal regenerative tissue (*Voutilainen et al* 1967) as the growth rate is completely different The more precise reasons for this require further study

After subtracting the mitotic indexes of each irradiation group from the mitotic index for the control group and comparing the result with the difference for the X ray group by giving as the ratio the difference of the X ray group/difference of the irradiated group the figures in Table 1 were obtained as the RBE value for the X ray group The other RBE values were smaller

The RBE of high energy radiation was smaller in all the groups than in the earlier work RBE was 0 81±0 19 in the group given cobalt irradiation compared with 0 90±0 26 for normal regenerative tissue It was 0 48±0 17 in the 20 MeV electron group and 0 52±0 22 in the earlier study These values were fairly similar in both investigations and the RBE value for cobalt was slightly higher than that arrived at by *Linden* (1964) in his Walker cancer study The RBE value obtained with 35 MeV electrons extremely low only 0 38±0 20 compared with 0 64±0 31 in the earlier work The difference was fairly great

The RBE values at some other time may differ because of the possible variations in the mitotic index and thus the values obtained at a given time do not warrant any final conclusions To be able to confirm the RBE values by administering different types of irradiation to the tumour tissue the post irradiation period before examination should be changed and a study should be made of the occurrence of mitoses as a function of the interval between the time of irradiation and the moment at which the biopsy specimen is taken

SUMMARY

Three month old CB stock white rats with Walker cancer were used for the experiments The tumour was implanted under the skin of the interscapular region The irradiation was given 10 days later The tumour dose was 300 rads and dose rate 40 rads/min The radiation was given by 200 kV X rays (HVL 1 6 mm Cu) cobalt 60 32 MeV photons of betatron and both 20 MeV and 35 MeV electrons of betatron The irradiation time was at 7 p m and the animals were decapitated 12 hours after irradiation Mitoses from the tumour were counted per 100 fields over an area of 0 785 sq mm

The mitotic counts were highest in the control group and lowest in the X ray group The RBE values for the different irradiation groups ranged from 1 to 0 38 when the RBE of the X ray group was 1 The

RBE of cobalt irradiation was 0.81±0.19, of 20 MeV electron 0.48±0.17, of 35 MeV electron 0.38±0.20 and photons 0.61±0.17.

It seems that different types of irradiation have different effects at a certain point of time, in the present work 12 hours after the irradiation. One reason obviously is the different changes in the mitotic cycle and lengthening of cell division.

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RECURRING DIGITAL FIBROUS TUMOR OF CHILDHOOD

I. Clinical and Morphological Aspects of a Case

By

Johan Ahlqvist, Pirkko Pohjanpelto, Lars Hjelt and Kari Hurme

Received 20.xii.66

A group of fibrous tumors with several features in common and called "recurring digital fibrous tumors of childhood" was recently described by *Reye* (1965). These often multiple and sometimes congenital tumors occurred in fingers and toes of children under 4 years of age. The growth of the tumors was comparatively slow, but they recurred unless radically removed. The feature that distinguishes them from other fibrous tumors is the presence of cytoplasmic inclusion bodies. This observation led Reye to suggest virus etiology. As far as we know, this tumor has not been reported outside Australia.

This paper describes a case diagnostized in Finland. The results of the virological studies on the tumor tissue will be dealt with in a subsequent paper (*Pohjanpelto et al.* 1967).

CLINICAL PICTURE

The patient, a girl, was born to healthy parents in May 1965 in Hameenlinna, southern Finland. The pregnancy and labors were uneventful. There were two older healthy children in the family.

A reddish spot was said to have been present on the third digit of the left hand at birth. Two months later this spot started to grow and a similar spot appeared on the fifth digit of the same hand. At 4 months the distal part of the third finger had been converted into an erythematous tumor and a small biopsy was taken. After the biopsy the tumor started to grow rapidly, the spot on the fifth finger swelled and an erythema appeared at the tip of the fourth finger. In spite of a rather benign biopsy report, the clinical picture at 6 months prompted surgical treatment and the two distal phalanges of the third and the fifth finger were amputated together with the erythematous, slightly swollen distal part of the fourth digit. A month later the stump of the third finger became erythematous, started to swell and reamputation had to be performed. Meanwhile, the pathologists had become familiar with the report of *Reye* (1965) and at 8 and 9 months tissue removed from erythematous scars and from a rarified area in the bone close to the tip of an amputated digit was sent for virological examination.

MORBID ANATOMY

General Description

The amputation specimen from the large tumor in the third digit

Aided by a grant from the *Sigrid Juselius Foundation*.



Fig 1

The proximal part of the amputated third digit which was converted int a tumor The distal interphalangeal j int and the nail are visible Scale in millimeters H&E



Fig 2

Alterati ns pre ent at the epidermal dermal juncti n at th e sit f the ampu tati n specimens in which actual tum r wa n t yet pre t H&E

is shown in Fig 1 The extent of the process can be seen since the distal interphalangeal joint and the nail are visible

In the fourth finger which clinically was erythematous and slightly swollen the rete ridges of the epidermis were elongated and surrounded by lymphocytes and histiocytes The epidermal alterations seemed to start with an intracellular probably cytoplasmic vacuolation The rete



Fig 3

Left Tumor tissue similar to the one on the dorsal aspect of the digit in Fig 2 Hematoxylin van Gieson *Right* Tumor cells from the same section at higher magnification

ridges seemed later to loosen up into a reticulum containing in its meshes cells with small condensed nuclei (Fig 2 left to right) In the deeper parts of the specimen around Krause corpuscles associated with myelinated nerves there were strands of cells and fibrils which resembled nerves but contained no myelin sheaths

On the volar side of the third digit (Fig 1) there were alterations resembling those in fourth digit described above On the dorsal and lateral aspects there was a highly cellular tissue reaching from the epidermis to the bone The tissue was intimately associated with the bone In Fig 1 the tissue actually erodes the bone under the proximal part of the nail The elongated fibroblast like cells and collagen bundles formed interdigitating bundles (Fig 3 left) The tumor cell nuclei were rather large with 1–2 small nucleoli and a delicate chromatine network without any prominent margination of the chromatin (Fig 3 right) Some regular mitotic figures were seen Some lymphocytes and rather many mast cells were present in the tumor tissue

In the original report on the specimens to the clinician the tumor was said to bring processes such as dermatofibromas neurofibromatosis and fibromatoses into mind and we did not arrive at a definite

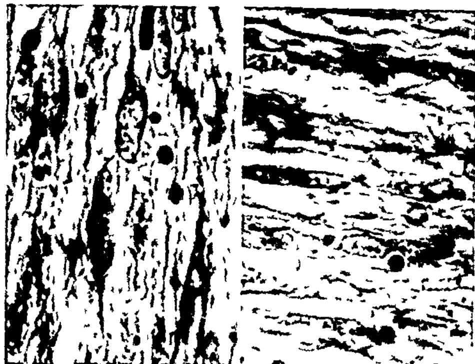


Fig. 4.
Inclusions in sections from the tumor overstained with toluidine blue. In the right picture one round dark inclusion seems to have formed an impression into the nucleus.

diagnosis until Reye's report appeared. Until then we had seen no inclusions.

Inclusions

In sections stained with our H&E a few faintly eosinophilic inclusions were seen in the tumor tissue. Only formalin fixed and paraffin-embedded material was available. When the intensity of the eosin was increased the number of inclusions seemed to increase. The inclusions stained orthochromatically with strong solutions of toluidine blue (Fig. 4). They were easily distinguished from other tissue components in sections overstained with eosin, briefly washed in water and dilute acetic acid and thereafter stained in a strong neutral solution of toluidine blue followed by rapid dehydration. In such sections the inclusions had a dark center surrounded by a rose rim.

The inclusions did not stain for 1,2-glycol groups with Schiff's reagent after oxidation with periodic acid. If not stated otherwise the following histochemical tests have been adapted from methods dealt with by *Pearse* (1960). The inclusions showed no alcohol stable metachromasia with toluidine blue. In a primitive "toluidine blue extinction test" (0.001 per cent solution in citrate buffers at pH 3, 4, 5 and 6 and

in tris buffers at pH 7 8 and 9 staining time 18 hours at 4 C examination in the staining solution) mast cells stained well nuclei nucleoli and the cytoplasm of the tumor cells poorly at pH 3 All these structures stained well at pH 4 but the inclusions did not stain well until at pH 7 in tris buffer In this solution almost all structures were metachromatic the metachromasia was not alcohol stable They gave a fairly strong tetrazonium reaction with Fast Blue B at pH 9 2 They did not stain with Scarlet Red for lipids With Sudan Black B they gave a weak positive reaction weaker than the reaction of for instance erythrocytes

The inclusions did not give a positive Feulgen reaction With a methyl green pyronine reaction carried out according to *Burck* (1966) the inclusions were not found to be pyroninophilic in contrast to the nucleoli and the cytoplasm of the tumor cells The orthochromatic basophilia of the inclusions was not noticeably influenced by digestion with ribonuclease (1 mg/ml at 37 C for 2½ hours) or by extraction with 10 per cent perchloric acid for 16 hours at 4 C These treatments removed the cytoplasmic basophilia of the tumor cells and of plasma cells in control sections

Some of the larger inclusions bore some resemblance to red blood cells but unlike the latter they did not stain for benzidine peroxidase

COMMENTS

Clinical Picture

Both clinically and morphologically the case corresponds to the cases described by Reye as recurring fibrous digital tumors of childhood

As far as we know this is the first case described outside Australia this seems to indicate that the tumor is not confined to Australia It is possible that it once recognized will not prove to be extremely uncommon as has been the case with so many other tumors

If these tumors are not extremely uncommon and if they continue to grow for a long time unless radically removed it seems that they should constitute a recognized syndrome in older patients Is it possible that they may undergo spontaneous regression? It would be interesting to obtain information about cases which have been left untreated

Morphology

In our case the process seemed to start with an erythema that corresponded morphologically to certain alterations in the epidermis and the upper dermis In these parts there were no clear inclusions nor any reticular degeneration of epidermal cells but it still seems that these alterations may be of some significance According to Reye the tumor tissue was separated from the epidermis by layer of normal collagen

Our studies suggest that the inclusions consist at least for the major

part of protein Carbohydrate containing compounds and lipids are probably present in very small amounts if at all The material seems to contain relatively few acidic groups According to Reye the inclusions were pyroninophilic probably containing RNA but we have not been able to confirm the presence of RNA by any of the tests performed and it thus seems that in our case the inclusions either do not contain RNA or that they contain it in such a form that we cannot demonstrate it

SUMMARY

A case diagnostized as recurring digital fibrous tumor of childhood is reported from Finland The process may possibly start with certain alterations in the epidermis or at the epidermal dermal junction Histochemical tests do not indicate the presence of RNA in the tumor cell inclusions

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RECURRING DIGITAL FIBROUS TUMOR OF CHILDHOOD

2 *Isolation of a Cell Transforming Agent*

By

PIRKKO POHJANPELTO JOHAN AHLQVIST KARI HURME and LARS HJELT

Received 22 xi 66

The presence of inclusion bodies in the cells of recurring digital fibrous tumors of childhood led *Reye* (1965) to suggest that these tumors might have a virus etiology From the recurrent tumor of a case described in part 1 (*Ahlqvist et al* 1967) of this study a cell transforming agent has been isolated

METHODS

The pieces of tissue sent for virological examination were ground with sterile sand in a mortar centrifuged at a low speed and the supernatant was inoculated into cultures of primary human fibroblasts and continuous human amnion cells U cells (*Pohjanpelto* 1961) The cultures were incubated at 34 C and 37 C the medium for fibroblasts was Eagle s medium containing 3 per cent horse serum and 5 per cent tryptone broth and that for U cells Eagle s medium containing 5 per cent calf serum

OBSERVATIONS

In the U cell cultures inoculated with the specimen cells detached from the glass in one to two weeks but in the following passages no such effect was observed In cultures of fibroblasts areas of tightly packed cells of characteristic fibroblast morphology in random array developed These areas grew in thickness and were transformed into thick clumps of cells visible to a naked eye Later the clumps were often detached from the glass leaving holes which soon filled with newly formed fibroblasts The clumping effect could be transferred to new cultures of fibroblasts suggesting that the agent responsible for it was able to multiply in primary human fibroblasts So far 10 successful passages have been made using either c[illegible]ls or concen[illegible]rated supernatant as the inoculum The response of the cells to infection varied to some extent Sometimes focuses of roun[illegible]ed cells r[illegible]embling epithelial cells appeared at the initial stage b[illegible] [illegible] formation of clumps (Fig 1) Sometimes the whole cell lay[illegible]r in[illegible]eased [illegible] thickness

Aided by grants from the *Sigrid Juselius Fo[illegible]lat[illegible]* [illegible] *nal Research* *[illegible]uncil for M[illegible]d[illegible]al Sci[illegible]n[illegible]s*

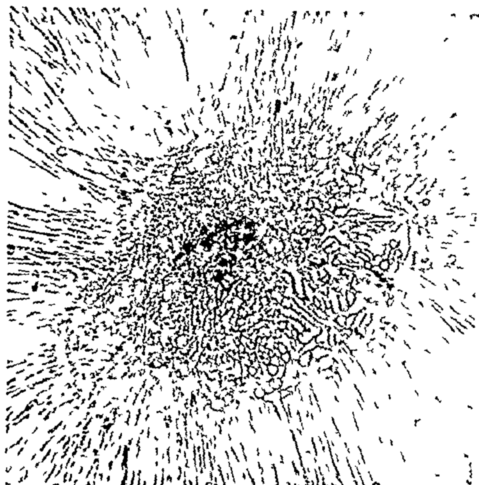


Fig. 1

Focus in a culture of primary human fibroblasts infected with the cell transforming agent

showing microscopically a characteristic pattern of alternate thick and thin areas (Fig. 2). Usually there was a marked change in the colour of the indicator in the medium during the first few days after inoculation as compared to the control, showing increased acidity, apparently due to increased cellular metabolism.

To obtain an idea of the size of the cell transforming agent, medium from over the infected cells was collected daily, stored at −70 C, filtered through a Jena glass sinter G5 able to retain bacteria and concentrated about 20 fold by dialyzing in a vacuum. The filtration through a bacterial filter did not destroy the cell transforming ability of the agent, indicating that it is smaller than bacteria and is possibly a virus.

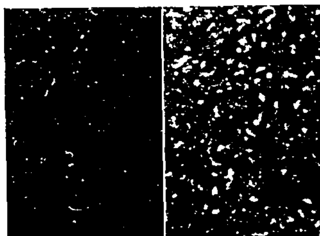


Fig 2
Primary human fibroblasts uninfected left and infected with the cell transforming agent right

DISCUSSION

It is not likely that the cell transforming agent isolated from the tumor is a laboratory pick up because isolation from the original specimen was made three times The small size of the agent shown by the filtration experiments indicates that it may be a virus In our present state of knowledge we do not know whether it is the cause of the tumor but the ability of the agent to transform cells is consistent with this idea

SUMMARY

An agent passing through bacterial filters and able to transform primary human fibroblasts *in vitro* has been isolated from a case of recurring fibrous digital tumor of childhood

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INVESTIGATIONS ON ANIMAL *STAPHYLOCOCCUS AUREUS* STRAINS

2 *Antigens*

By

JEAN LOUIS MARANDON and PER OEDING

Received 13 x 66

Relatively few studies have been made on the antigenic structure of animal *Staphylococcus aureus* strains. The results indicate that *Staph aureus* strains of human and animal origin have shared antigens but that there are quantitative as well as qualitative differences. Frequently the only criterion that the strains examined were true animal strains has been their isolation from animals or their surroundings, whereas the identity has not been ascertained by biochemical tests.

In a previous paper (8) a material of coagulase positive animal *Staph aureus* strains was examined and classified by biochemical methods. Strains producing beta hemolysin but not fibrinolysin were considered to be true animal staphylococci and were selected for serologic study. In the present report these animal strains have been examined for the presence of human *Staph aureus* antigens.

MATERIAL AND METHODS

The material consisted of 120 *Staph aureus* strains classified as animal strains (8). Eighty four per cent of the strains were isolated from bovine mastitis pus, the others from animals other than the cow.

Agglutination was performed on slides using live bacteria and 11 human factor sera according to the method of *Oeding*. For technical details see *Haukenes et al.* (3).

Agar gel diffusion was performed as described by *Haukenes & Oeding* (2). Eighteen hr live nutrient agar cultures of the strains were tested against whole immune sera of human *Staph aureus* strains Wood 46, 263 and Cowan I. Teichoic acid, polysaccharide 263 and protein A were included as references (12, 4).

RESULTS

Human Staph aureus *agglutinogens. Seventy eight* (6[illegible] per cent) of the animal strains were typable in factor sera against human *Staph aureus* agglutinogens (Table 1). Forty strains did not agglutinate in any factor serum whereas two strains agglutinated spontaneously. The typable strains had a wide range of antigenic patterns. Their content of human staphylococcal agglutinogens seemed to be somewhat low, as 59 of the

strains had only one or two of the antigens and the agglutinations were often weak The *h* antigen was the commonest It was found in 30 strains which agglutinated strongly in *h* serum (Table 2) In 21 strains the *h* antigen was found alone

TABLE 1

Agglutination of 120 Animal Strains in 13 Human Staph aureus *Factor Sera*

	Strains No	%
Typable	78	65
Not typable	40	33
Spont. aggl	2	2

TABLE 2

Distribution of Agglutinogens in the 78 Typable Strains

Agglutinogen	Strains No	%
a_1	6	8
a_5	14	18
b_1	5	6
c_1	19	24
e	4	5
h_1	1	1
h	30	39
i	17	21
k_1k	4	5
m	3	4
n	2	3
263-1	4	5
263-2	12	15

TABLE 3

Human Staph aureus *Group Precipitinogens Present in 120 Animal Strains*

	Strains No	%
Polysacch A	103	86
Polysacch 263	5	4
Protein A	54	45

Human Staph aureus *precipinogens* Table 3 illustrates the distribution of three human *Staph aureus* group precipitinogens in the 120 animal strains One hundred and three strains gave a strong precipitation line against serum Wood 46 corresponding with standard polysaccharide A Polysaccharide 263 was only demonstrated in five strains

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By

JEAN LOUIS MARANDON and PER OEDING

Received 13 x 66

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were obtained by *Pillet et al* (10) and *Mercier et al* (9) who used the three Cowan sera for typing *Malik & Singh* (7) and particularly *Live & Nichols* (6) were able to type a high percentage of their animal strains in human factor sera *Grun* (1) and *Pulverer* (11) concluded that animal strains were much less often typable in human factor sera than human strains and if typable they had fewer and weaker agglutinogens The fact that a number of strains are untypable would indicate that animal staphylococci have agglutinogens not represented in the human typing set One new antigen has been demonstrated in this investigation *Grun* (1) described a new agglutinogen (*l*) in an animal strain and this antigen was also demonstrated in human strains Investigations by *Ishii* (5) and *White et al* (13) also indicate that animal staphylococci have specific antigens in addition to the agglutinogens shared with human strains For type differentiation of animal staphylococci and their distinction from human strains it is important that more work be done on the specific animal antigens

The examination of our animal strains for the presence of human *Staph aureus* group precipitinogens showed that they have the same type of antigenic ribitol teichoic acid as the cell walls of human strains Beta linked N acetylglucosaminyl residues (polysaccharide A) were regularly demonstrated whereas alpha linked N acetylglucosaminyl residues (polysaccharide 263) seemed to be infrequent in our material The wall also have protein A

With regard to group precipitinogens as well there is no principal difference between animal and human *Staph aureus* These antigens seemed however to be less readily demonstrable in the animal strains either because they are not present in all strains or because the quantities are small In human *Staph aureus* strains the group precipitinogen show great quantitative variations and absorptions have not been carried out to exclude the presence of small amounts in the animal strains which appeared to lack the antigens

SUMMARY

One hundred and twenty animal *Staph aureus* strains which had been isolated mainly from bovine mastitis pus and were identified biochemically have been examined serologically Sixty five per cent of the strains were typable in human *Staph aureus* factor sera The typable strains had fewer and weaker agglutinogens than human strains The animal strains also had the same group precipitinogens as human staphylococci A specific antigen was demonstrated Animal *Staph aureus* strains therefore seem to have many of the same antigens as human *Staph aureus* strains although in smaller amounts and in addition certain specific antigens

whereas 54 strains produced lines which corresponded with protein A An interesting result was that 11 of these strains exhibited only a single line and not the double protein A line regularly observed in human *Staph aureus* strains against Cowan I immune serum The line corresponding to that produced with human serum could not be demonstrated in these strains When however crude protein A was prepared from two of the strains both antigens were present

The majority of the animal strains also gave other non identified precipitation lines with the three human *Staph aureus* immune sera

No correlation was found between human staphylococcal phage groups and the presence of group precipitinogens It was however remarkable that 15 out of the 17 strains in which polysaccharide A was not demonstrated were not typable by phage

Specific animal Staph aureus *antigens* Rabbit immune sera were produced against three representative animal strains in which none of the human *Staph aureus* agglutinogens had been demonstrated The unabsorbed sera agglutinated all our human *Staph aureus* type strains The sera diluted 1 10 were then absorbed with representatives of the human type strains in order to remove all known human agglutinogens After absorption none of the human type strains agglutinated in the three sera Two of the absorbed sera had also been exhausted for antibodies against the homologous strains whereas the third serum still agglutinated the homologous strain and 16 other animal strains After concentration of this serum an unknown precipitation line was produced on agar gel against the homologous strain and 12 of the animal strains which had been positive on agglutination

DISCUSSION

The 120 strains which have been submitted to serological investigation are independent and according to biochemical tests true animal strains (8) However they are mostly bovine strains isolated from mastitis pus and therefore not representative of animal strains in general The typing with human *Staph aureus* factor sera shows that specific human agglutinogens are also quite widely distributed in animal strains It is possible that certain human *Staph aureus* agglutinogens are more frequently encountered in animal strains than others Thus antigen *h* was quite frequent and strong in our material But the results also show that there is a clear difference between animal and human strains with regard to the content of human agglutinogens While nearly 100 per cent of human *Staph aureus* strains are typable in the set of human factor sera only 65 per cent of this material of animal strains could be typed In addition the typable animal strains generally gave weaker agglutinations and had fewer agglutinogens

Other authors have been more or less successful in their attempts to type animal strains with human *Staph aureus* sera Very poor results

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A NEW PNFUMOCOCCUS TYPE

Type 12A

By

ERNA LUND and AGNETE MUNKSGAARD

Received 18 x 66

The number of types given in the latest publication on serological classification of pneumococci is 81 (*Lund* 1962) a new type 12A is described here In May 1965 an organism 411/65 was isolated from the blood of a 75 years old man This strain manifested the usual cultural characteristics of a Pneumococcus and gave distinct capsular reactions in diagnostic pneumococcal sera (*Lund* 1963) of both type 12 and type 46 and moreover a weak reaction in type 44 serum Further 3 strains giving similar reactions were subsequently isolated at Statens Serum institut

6 months after isolating the first of these organisms two strains of pneumococci which reacted in sera of types 12 44 and 46 were sent to us by bacteriologist *David Hansman*[1] (Sydney) and during the fol lowing months 7 more similar strains were received from Australia giving a total of 14 strains

These 14 strains were found to be Gram positive diplococci soluble in bile sen sitive to optochin growing aerobically as smooth colonies on 5 or 10 per cent blood agar showing α hemolysis and growing homogeneously in serumbroth

SENSITIVITY

Tested by the tablet method (*Lund* 1965) the 14 strains showed identical sensitivity to antibacterial drugs being fully sensitive to penicillin sulphonamides tetra cyclines erythromycin bacitracin chloramphenicol novobiocin methicillin and nitrofurantoins moderately sensitive to streptomycin neomycin and fucid n and resistant to pol myxin and colimycin

VIRULENCE

3 of the strains 411/65 89 6/65 and 11743/65 showed h gh irulence to mice

BIOCHEMICAL REACTIONS

None of the 14 strains fermented arabinose xylo ul t ros tol sorbitol mannitol or inulin in 8 days All strains fermented glu (th ut gas) galactose lactose sucrose maltose aesculin and salicin on the f st d (M h – *Lund* 1949)

[1] Our best thanks to bacteriologist *David Hansma* Pathol g Department The Women s Hospital Sydney Australia for the tra ns and for g od collaboration

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only react in the omni serum pooled serum E group serum 12 and factor serum 12c In Table 2 it is seen that type 12 reacts in the same sera except in factor serum c but in stead it reacts in factor serum 12b

CROSS ABSORPTION

Cross absorption tests between the types 12 44 46 and the new type 12A (Table 3) showed that type 12A was so closely related to type 12 that it was practical to put both types into one group 12 (pn 12 + pn 12A)

TABLE 3

Cross Absorption of the Types 12 12A 44 and 46

sera	antigenic formulas of the sera	capsular titers of the sera with types 12	12A	44	46
12 not absorbed	12a 12b 12d	512	256	16	0
12 absorbed with 12A	12b	16	0	4	0
12 absorbed with 44	12a	128	128	0	0
12 absorbed with 46	12a 12b 12d	128	128	8	0
12A not absorbed	12a 12c 12d	256	512	32	16
12A absorbed with 12	12c	0	16	0	8
12A absorbed with 44	12a 12	128	256	0	16
12A absorbed with 46	12a 12d	256	256	16	0
44 not absorbed	44a 44b 12b 12d	32	32	256	32
44 absorbed with 12	44a 44b	0	0	128	32
44 absorbed with 12A	44a 44b 12b	16	0	128	16
44 absorbed with 46	44a 12b 12d	16	8	64	0
46 not absorbed	46a 12c 12d	0	32	32	128
46 absorbed with 12	46a 12c 12d	0	16	16	128
46 absorbed with 12A	46a 44b	0	0	16	128
46 absorbed with 44	46a 12c	0	16	0	64

type	*antigenic formulas*
12	12a 12b 12d
12A	12a 12c 12d
44	44a 44b 12b 12d
46	46a 12c 44b

From the cross absorption tests the antigenic formula for type 12A can be given as 12a 12c 12d but the establishment of this new type makes it necessary for the formulas for three of the previously known types to receive an additional antigen (Tabl[illegible]) The [illegible] 12d has been added to types 12 and 44 and 12c to type 46

Type 12 12a 12b *12d*
Type 44 44a 44b 12b *12d*
Type 46 46a *12c* 44b

ANTIPNEUMOCOCCAL SERUM

Antipneumococcal serum was prepared from the original strain 411/65 by immunizing ten rabbits (*Lund* 1960). The strain 411/65 gives capsular reactions and agglutination in unabsorbed sera of pneumococcal types 12, 44 and 46, but not in sera of the remaining 78 types. The titers of the reactions are given in Table 1.

TABLE 1

Cross Reactions (Capsular Titers) of the Pneumococcal Types 12, 12A, 44 and 46

unabsorbed sera	capsular titers with strains			
	12	12A	44	46
12	512	256	16	0
12A	256	512	32	16
44	32	32	256	32
46	0	32	32	128

Three batches of type 12 serum showed identical cross reactions and nearly the same titers (capsular and agglutination) with the new type 12A. Two batches of type 44 serum and two of type 46 also had identical cross reactions with type 12A. The most potent sera of the types 12, 44 and 46 were chosen for the cross absorption tests. No cross reactions other than those given in Table 1 were found in the sera mentioned.

DIAGNOSTIC SERA

A diagnostic serum is made specific (for the type, group or pool) by absorption with the heterologous types. Entering the new type 12A into the system of diagnostic sera, types 12 and 12A are gathered into group 12, the diagnostic serum for type 12 reacting with both types in this group (Table 2).

TABLE 2

Diagnostic Pneumococcal Sera Reacting with Group 12 (types 12 and 12A)

diagnostic sera	capsular titers with strains	
	type 12	type 12A
group 12	64	32
pooled serum F	32	32
omni serum	16	16
factor serum 12b	16	–
factor serum 12c	–	16

The diagnostic sera for the types 44 and 46 must be absorbed with type 12A to be typespecific. After absorption a strain of type 12A will

only react in the omni serum pooled serum F group-serum 12 and factor serum 12c In Table 2 it is seen that type 12 reacts in the same sera except in factor serum c but in stead it reacts in factor serum 12b

CROSS ABSORPTION

Cross absorption tests between the types 12 44 46 and the new type 12A (Table 3) showed that type 12A was so closely related to type 12 that it was practical to put both types into one group 12 (pn 12 + pn 12A)

TABLE 3
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12 absorbed with 44	12a	128	128	0	0
12 absorbed with 46	12a 12b 12d	128	128	8	0
12A not absorbed	12a 12 12d	256	512	32	16
12A absorbed with 12	12c	0	16	0	8
12A absorbed with 44	12a 12c	128	256	0	16
12A absorbed with 46	12a 12d	256	256	16	0
44 not absorbed	44a 44b 12b 12d	32	32	256	32
44 absorbed with 12	44a 44b	0	0	128	32
44 absorbed with 12A	44a 44b 12b	16	0	128	16
44 absorbed with 46	44a 12b 12d	16	8	64	0
46 not absorbed	46a 12c 12d	0	32	32	128
46 absorbed with 12	46a, 12c 12d	0	16	16	128
46 absorbed with 12A	46a 44b	0	0	16	128
46 absorbed with 44	46a 12	0	16	0	64

type	*antigenic formulas*
12	12a 12b 12d
12A	12a 12c 12d
44	44a 44b 12b 12d
46	46a 12c 44b

From the cross absorption tests the antigenic formula for type 12A can be given as 12a 12c 12d but the establishment of this new type makes it necessary for the formulas for three of the previously known types to receive an additional antigen (Table 4) The antigen 12d has been added to types 12 and 44 and 12c to type 46

Type 12 12a 12b *12d*
Type 44 44a 44b 12b *12d*
Type 46 46a *12c* 44b

TABLE 4

Danish Type Designations of 82 Pneumococci
Antigenic Formulas According to *Kauffmann* and *Lund*

type	antigenic fomulas	type	antigenic fomulas
1	1a	20	20a 20b 7g
2	2a	21	21a
3	3a	22	22a 22b
4	4a	22A	22a 22c
5	5a	23	23a 23b 16b
6A	6a 6b	23A	23a 23c 15c
6B	6a 6c	23B	23a 23b 23d
7	7a 7b	24	24a 24b 24d 7h
7A	7a 7b 7c	24A	24a 24c 24f
7B	7a 7d 7e 7h	24B	24a 24b 24e 7h
7C	7a 7d 7f 7g 7h	25	25a 25b
8	8a	27	27a 27b
9A	9a 9c 9d	28	28a 28b 16b 23d
9F	9a 9b 9c 9f	28A	28a 28c 23d
9N	9a 9b 9e	29	29a 29b 13b
9V	9a 9c 9d 9g	31	31a 20b
10	10a 10b	32	32a 27b
10A	10a 10c 10d	32A	32a 32b 27b
11	11a 11b 11e 11g	33	33a 33b 33d
11A	11a 11c 11d 11e	33A	33a 33b 33d 20b
11B	11a 11b 11f 11g	33B	33a 33c 33d 33f
11C	11a 11b 11c 11d 11f	33C	33a 33c 33e
12	12a 12b 12d	34	34a 34b
12A	12a 12c 12d	35	35a 35b 34b
13	13a 13b	35A	35a 35c 20b
14	14a	35B	35a 35c 29b
15	15a 15b 15c 15f	35C	35a 35c 20b 42a
15A	15a 15c 15d 15g	36	36a 9e
15B	15a 15b 15d 15e 15h	37	37a
15C	15a 15d 15e	38	38a 25b
16	16a 16b 11d	39	39a 10d
17	17a 17b	40	40a 7g 7h
17A	17a 17c	41	41a 41b
18	18a 18b 18c 18f	41A	41a
18A	18a 18b 18d	42	42a 20b 35c
18B	18a 18b 18e 18g	43	43a
18C	18a 18b 18c 18e	44	44a 44b 12b 12d
19	19a 19b 19d	45	45a
19A	19a 19c 19d	46	46a 12c 44b
19B	19a 19c 19e 7h	47	47a 35a 35b
19C	19a 19c 19f 7h	48	48a

The formulas valid up to now are given for comparison (*Kauffmann Lund & Eddy* 1960)

Type 12 12a 12b
Type 44 44a 44b 12b
Type 46 46a 44b

All 14 strains of type 12A gave identical capsular reactions in the sera used for the cross absorption tests mentioned in Table 3 the stronger antigens in these 14 strains must therefore be identical

Types 12A and 46 have been found to be highly virulent to mice whilst types 12 and 44 show only moderate virulence (*Lund* 1950). Types 12A and 46 have the antigen 12c in common but it is missing from types 12 and 44; it is therefore possible that this antigen is responsible for the higher virulence.

TABLE 5

14 Strains of Type 12A from 13 Patients

strain	patient	years of age	isolated from	clinical diagnosis
1 411/65	♂	76	blood	bronchopneumonia septicæmia
2 527/66	♀	62	blood	bronchopneumonia septicæmia
3 552/65	♀	69	blood	abscessus cerebri bacteriæmia
4 559/66	♂	21	blood	pneumonia duplex bacteriæmia
5 8956/65	♂	67	sputum	bronchopneumonia
6 11136/65	♂	70	conjunctiva	bronchit. chron.
7 11743/65	♂	82	sputum	bronchit. chron.
8 11793/65	♂	76	sputum	bronchit. chron.
9 12486/65	♂	54	sputum	bronchopneumonia
10 10392/65 10394/65	♂	61	clavicula meninges	osteomyelitis meningitis
11 6412/66	♂	[illegible]6	sputum	bronchit. chron.
12 6365/66	♂	82	sputum	bronchit. chron.
13 7197/66	♂	22	sputum	bronchit. chron.

The sources of the strains are given in Table 5. Most of the strains were isolated from elderly patients and predominantly from men: of 11 male patients only three were less that 60 years of age and the 2 women were both in their sixties.

One of the strains (11136/65) was isolated from a conjunctival swab taken prior to operation on a cataract in a patient with chronic bronchitis. One patient died from meningitis caused by Pn. 12A (strain 10394/65) following a motor accident, the same type (strain 10392/65) having been isolated from an osteomyelitis of his clavicula.

SUMMARY

A new pneumococcal type 12A is described bringing the total number of types to 82. The new type reacts with the types 12, 44 and 46. It is put up as type 12A forming a group 12 with type 12.

14 strains of type 12A from 13 patients have been isolated in Denmark and Australia

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PRODUCTION OF CHLAMYDOSPORES BY *CANDIDA CLAUSSENII*

By

J Brown Thomsen

Received 14 xii 66

A new species *Candida claussenii* was created by *Lodder & Kreger van Rij* in 1952 based on the micro and macromorphological deviations from their description of *Candida albicans* (1952) Like *Stelling Dekker* (1931) they found that the physiological reactions of the strain (received in 1910 from Claussen in Copenhagen labelled *Saccharomyces pastorianus*) was in agreement with those of *Candida albicans*

The morphological characteristics on which the species was created were the absence of ball like clusters of blastospores on the pseudomycelium the incapability of producing chlamydospores a more elongated form of the blastospores after cultivation in malt extract and further the pseudomycelium never develops into the agar as it does in *Candida albicans*

van Uden et al (1956/58) published some pathogenicity experiments with the type strain of *Candida claussenii* They found that its pathogenicity was of the same order as that of *Candida tropicalis* Without further comments they described a negative fermentation of galactose (a negative galactose fermenting ability was also mentioned in a previous report by the same authors in 1956) and in their quotation of the species description by *Lodder & Kreger van Rij* (1952) they wrongly reported the galactose fermentation as negative

A negative galactose fermentation is also reported by *Kocková Kratochvilova et al* (1963) in their Table 1 which gives a review of common and distinguishing features of *Candida albicans* *Candida stellatoidea* and *Candida claussenii* In their Table 3 and in a later report (*Kocková Kratochvilova* 1964) the fermentation of galactose by *Candida claussenii* is reported as being positive In 1965 the Centraal bureau voor Schimmelcultures still described the galacto[illegible] [illegible] of the type strain is positive (*Slooff* personal communication)

Sanchez Marroquin (1958) found further similarities [illegible] *Can*

Supported by grant [illegible] the *Danish National Research F*[illegible]

I wish to thank the [illegible]hoslovak collection [illegible] for the strains generou[illegible] [illegible]lied and the C[illegible] aalbure[illegible] cultures for the type strain

dida albicans and *Candida claussenii* as to antibiotic sensitivity, the reduction of sulphites and the lack of hydrolysis of urea and tyrosine and concluded that these species may prove to be synonymous.

Artagaveytia Allende & Legnani (1962) and *Artagaveytia Allende* (1965) accepted the synonymity with reference to previous investigations by the above mentioned authors. *Fragner* (1962) still accepted *Candida claussenii* as a separate species with mere reference to the publication by *Lodder & Kreger van Rij* (1952).

In all of the publications on *Candida claussenii* previously mentioned it is accepted that this species is not able to form chlamydospores.[1]

This paper describes the observation of chlamydospore formation in *Candida claussenii*, experiments on the serum tube formation ability and some physiological properties.

MATERIALS AND METHODS

Strains. Four strains of *Candida claussenii* were used. Three one cell cultures were made from each strain using the technique of *Orskov* (1922). The properties of the four original strains and of the twelve cloned cultures were alike, three of the original cultures being the type strain of the species.

The four strains were the following:

1. Type strain of *Candida claussenii* CBS No. 1949.
From the Czechoslovak collections of Microorganismus:
2. (CHI SAS) 29-31-1 = IFO 0759 1961 = CBS 1949
3. (CHI SAS) 29-31-2 = CBS 1949
4. (CHI SAS) 29-31-3

During the experimental period the strains were kept in our collection on malt extract agar slants and transferred every third month.

Chlamydospore formation. Two media (*a* and *b*) were used to promote the formation of chlamydospores.

a. Rice medium with the following composition (*Taschdjian* 1953):

White rice	20 g
Special agar noble (Difco)	20 g
Distilled water	1000 ml

b. Same composition as medium *a* with Tween 80 in a 1 per cent concentration (*Taschdjian* 1957).

The cultures were scratched into a thin layer of the medium on slide covered with cover glasses and cultivated in a wet chamber (Petri dish) at room temperature which during the experimental period was 20–22° C. The cultures were examined on these media daily for 8 days.

Fermentation. The media were made principally according to the description of *Lodder & Kreger van Rij* (1952) with the following composition of the basic sugar free substrate:

Yeast extract (Difco)	14 g
Water	2520 ml

The sugars were added to a final concentration of 2 per cent. The sugars were heat treated for 2 × 20 min at 100° C. (*Lodder & Kreger van Rij* 1952 used 15 min

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1. At the Third International Symposium on Yeasts in Bratislava, July 1966, Dr. *Wickerham* (Peoria, Illinois, U.S.A.) informed me that he too had observed chlamydospores of the same form as described in this paper in a strain of *Candida claussenii*.

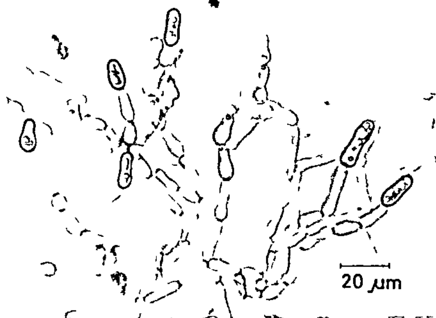


Fig 1

Candida claussenii Chlamydospores in Rice medium *a*

at 120 C) Table 1 shows the various sugars used Two different makes of galactose and sucrose were used

The production of gas was used as an indicator for the fermentation (Durham glasses)

The cultures were incubated at room temperature (20–?? C) The reactions were read daily for 10 days The inoculation was made by a tiny inoculum from malt extract agar cultures

Assimilation of carbon sources The reactions were performed on solid medium with the addition of yeast extract in plastic Petri dishes (not air tight) by the method used by *Lodder & Kreger van Rij* (1952)

With reference to *Wickerham & Burton* (1948) the carbon sources that were tested are shown in Table 2

The reactions (growth zones) were examined after cultivation at 25 C for 2 3 days and again after further cultivation for 2–3 days at 17 C

Rapid identification test for Candida albicans The filamentation reaction *(Reynolds & Braude* 1956 *Taschdjian et al* 1960 *Stenderup & Brown Thomsen* 1964) was performed in human serum in small tubes The serum was inoculated with yeast cells from a 2–3 day old malt extract agar culture (35 C) and incubated fo hours at 35 C. The yeast cells were then examined on slides for the p ence of characteristic filaments The activity of the serum was continu u ecked by the use of a typical *Candida albicans* strain

RESULTS

All cloned cells produced chlamydospores in approximately 2–3 days in medium *a* which did not contain Tween 80 Figs 1 and 2 show the

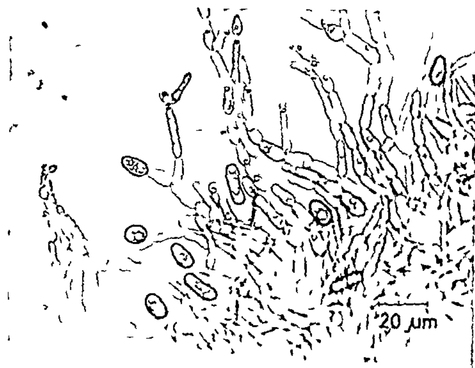


Fig 2
Candida claussenii Chlamydospores in Rice medium *a*

chlamydospores in this medium In medium *b* which contained Tween 80 in a 1 per cent concentration extremely few chlamydospores were observed

The results of the fermentation reaction are shown in Table 1

TABLE 1
Fermentation Reaction

d glucose	(Merck)	+
d-galactose	(Merck)	+
d-galactose	(Fluca)	+
d-maltose	(Merck)	+
d-sucrose	(Pflanstiehl)	(+)
d sucrose	(BDH Analar)	((+))
d lactose	(Merck)	—
inulin	(Merck)	—
raffinose	(Merck)	—

One pair of brackets indicates that the Durham glasses could not be completely filled in 10 days
Two pairs of brackets indicate that only a small bubble was formed.

The results of the assimilation reaction (auxanographic method) are shown in Table 2

TABLE 2

Auxanogram

d-glucose	(Merck)	+
d galactose	(Merck)	+
d-sucrose	(Pflanstiehl)	+
d maltose	(Merck)	+
d-lactose	(Merck)	—
l sorbose	(Merck)	+
d-cellobiose	(Sigma)	—
d trehalose	(BDH)	+
d-melibiose	(Pflanstiehl)	—
raffinose	(Merck)	—
d melezitose	(Merck)	—
inulin	(Merck)	—
d xylose	(Merck)	+
l-arabinose	(Merck)	+
d arabinose	(Pflanstiehl)	variable
d-ribose	(Sigma)	—
erythritol	(Merck)	—
adonitol	(Merck)	+
dulcitol	(Merck)	—
d-mannitol	(Merck)	+
d sorbitol	(Merck)	+
alfa methyl d glycosid	(BDH)	+
salicin	(G T Gurr)	—
lactic acid	(Merck)	+
succinic acid	(Merck)	+
citric acid	(Merck)	+
inositol	(Merck)	—

The filamentation reaction in serum was negative for all the strains The control strain of *Candida albicans* produced typical filaments

DISCUSSION

In the original description of the species *Candida claussenii* (*Lodder & Kreger van Rij* 1952) it was stated that the fermentation and assimilation reactions used for the description were like those for *Candida albicans* and that the properties characterizing *Candida claussenii* were the incapability of producing chlamydospores and the deviation from the accepted morphology of *Candida albicans* as mentioned in the introduction

The author agrees as to the similarities between the fermentation and assimilation reactions of *Candida albicans* and *Candida claussenii* mentioned in the original description (where fermentation of glucose g[illegible]actose sucrose maltose and lactose and assimilation of [illegible] galactose sucrose maltose and lactose were tested) With spe[illegible]erence to the diversities as regards the fermentation of galact[illegible]tioned in the introduction it is worth noticing that ferment[illegible] la tose was positive for both makes tested (Table 2)

The chlamydospores produced by these strains of *Candida claussenii* are dissimilar to those normally seen in freshly isolated strains of *Candida albicans* but according to *Wickerham* ([illegible]) and *Lodder &*

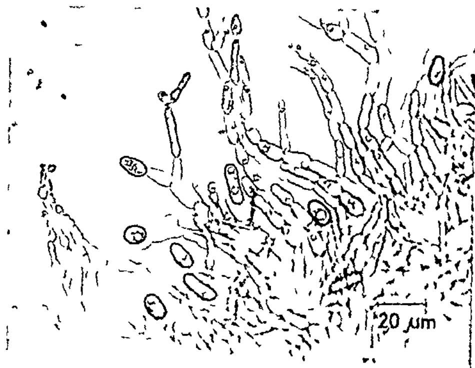


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d-sucrose	(Pflanstiehl)	(+)
d-sucrose	(BDH Analar)	((+))
d-lactose	(Merck)	—
inulin	(Merck)	—
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3 per cent Immidately after extraction the teeth were cut at a right angle to their length axis into two or three parts thereby obtaining a quicker and better fixation with the neutral formalin Decalcification followed in formic acid sodium citrate solution After double paraffin embedding the middle part of the root was sectioned at a right angle to the length axis of the tooth 6 8 μm thick sections and stained with haemalun eosin and periodic acid Schiff Each type of staining was performed in the same batches including both diabetic and nondiabetic material

Results In all the sections the peripheral third of the pulp showed only small vessels whereas the central part presented small and large vessels and nerves

In the PAS stained sections from diabetics PAS positive mural thickenings were seen in both small and large vessels These vascular changes seemed to be most pronounced in the central areas of the pulp but could be seen even in the peripheral part Similar changes could not be demonstrated in the control material

Calcifications in the pulp were found in diabetics and nondiabetics But in diabetics these calcifications were more frequent and were often sickle shaped and embracing the vessels In some areas the calcification was so marked that only remnants of vessels could be discerned

Discussion The present investigation shows that patients with diabetes mellitus of long duration present late diabetic manifestations in the form of vascular changes in the dental pulp identical with those observed in other tissues *i e* marginal gingiva and periodontal membrane (*Russell* 1966 1967) The higher frequency and peculiar shape of the calcifications in the diabetic dental pulp can hardly be connected with a contingent hypertension as they are often extramural

The two groups are yet too small to allow any definite conclusions in regard to the morphological variation of the calcifications An investigation to show how early and how often these alterations occur is in preparation

Conclusion A preliminary investigation shows that in patients with diabetes mellitus of long duration late diabetic vascular changes may be demonstrated in the dental pulp which in these patients also seems to exhibit a specific type of calcification

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THE ADOPTIVE TRANSFER OF EXPERIMENTAL MOUSE AMYLOIDOSIS BY INTRAVENOUS INJECTIONS OF SPLEEN CELL EXTRACTS FROM CASEIN TREATED SYNGENEIC DONOR MICE

By

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A number of investigations in recent years dealing with the pathogenesis of experimental amyloidosis has indicated the decisive role played by mesenchymal cells in particular by those belonging to the reticulo endothelial system (*Teilum* 1954 1956 1964 *Cohen et al* 1965) Observations of the histochemical events taking place during the induction of experimental amyloidosis together with studies of the influence of various enhancing and inhibiting factors on the dynamics of amyloid formation led to the concept of a biphasic local cellular formation of amyloid (*Teilum* 1964) In light of these premises it was considered a probability that the primary pyroninophilic phase of amyloid formation induced in one animal might be transferred to a second syngeneic animal with spleen cells thus without delay eliciting the second amyloid phase in the recipient Such experiments have proved successful thereby substantiating the crucial role of *cells* in the pathogenesis of experimental amyloidosis (*Werdelin & Ranlov* 1966 *Ranlov & Werdelin* 1967)

The following experiment aims at the elucidation of the nature of the factor responsible for the adoptive transfer of experimental mouse amyloidosis *in a syngeneic system—in particular at the question* whether its activity is related to the intact cell

MATERIAL AND METHODS

Animals Throughout the experiments the same stock of randomized closely inbred C3H mice 2–4 months of age were used Sex distribution was equal

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Experiment A

Donor mice. 25 randomly selected were treated with 17 daily casein injections 5 times a week according to the method originally described by *Kuczynski* (1922). Per injection 0.5 ml of a solution (5 per cent) of casein in 0.25 per cent NaOH was given subcutaneously in the back. This treatment resulted in minute amounts of amyloid in the spleens of approximately 1/3 of the donors. In all donor spleens a heavy pyroninophilia was evident. The day after the last injection all donor mice were killed by cervical dislocation and the spleens were harvested and pooled in cold isotonic saline. From each spleen a small specimen was fixed in neutral formalin for histological examination. The total yield of splenic tissue was 6.5 gm (wet weight.

Cell suspension. The spleens were gently teased apart in a loose fitting Potter Elvehjem homogenizer. After filtration twice through a metal sieve the suspension was washed twice in cold isotonic saline and finally made up to a suspension containing spleen cells equivalating 200 mg wet weight of splenic tissue per ml (corresponding to approximately 200×10^6 nucleated spleen cells per ml). 6 ml of this suspension (the equivalent of 1.2 gm wet weight of donor spleen) was kept for injection in the experimental group I (see below).

Cell homogenate and fractionations. Extracts of donor spleen tissues were made largely according to the method for preparing antigenic tissue extract described by *Medawar* (1963): the remaining cell suspension corresponding to an initial wet weight of pooled donor spleens of approx. 5.0 gm was centrifuged for 5 minutes at 2° C at 1 500×G. The supernatant saline was discarded and the cells resuspended in 10 ml cold isotonic saline to which 30 ml icecold distilled water was thereupon added under vigorous stirring. The cells, now sticky and osmotically swollen were spun down again at 1 500×G (2° C), cut up coarsely with scissors, resuspended in 40 ml of icecold distilled water and spun out again. The gelatinous cell mass was cut up again with scissors, resuspended in 17 ml distilled water and in volumes of 8.5 ml homogenized in a Waring blender at half speed for 5 minutes followed by top speed for further 2 minutes. After the removal of air bubbles the homogenate was finally exposed to ultrasonic irradiation for 45 seconds using an M.S.E. Ultrasonic Disintegrator, frequency 20 kc/s, output 60 W. The final result was 17 ml of a transparent yellowish fluid. In order to restore the original salt concentration 0.081 volume 2 M NaCl was added. This caused a heavy precipitate to form which was thereupon removed by centrifugation at 5 000×G for 10 minutes. This sediment will in the following be referred to as "DNA protein" while the corresponding supernatant will be referred to as "crude semisoluble extract" (*Medawar* 1963).

The sedimented fraction termed "DNA protein" was suspended in isotonic saline so that 0.5 ml of the suspension contained material representing an initial wet weight of 100 mg of the pooled donor spleens from which the preparation started. The final dry weight of "DNA protein" in 0.5 ml of this suspension was determined as 2.0–2.5 mg (after evaporation of water and deduction of the 4.5 mg NaCl). The dry "DNA protein" was microscopically an amorphous highly Feulgen positive material without recognizable cell structures. It may be considered made up of all the cellular DNA, DNA linked enzymes and histones (*Klenow* 1966).

The supernatant fraction termed "crude semisoluble extract" was likewise adjusted so that 0.5 ml represented an initial wet weight of 100 mg pooled donor spleen. The final dry weight in an aliquot of 0.5 ml was 2.5–3.0 mg. This dry material may be considered derived from mitochondrias, microsomes, various soluble enzymes and more than 90 per cent of the cell RNA (*Klenow* 1966).

Treatment of Recipients

Group I. 12 mice were given intravenously in the tail 0.5 ml of the original suspension of viable spleen cells from the casein treated donor mice. This dose corresponds to an initial wet weight of 100 mg pooled donor spleen and to approx. 100 × 10^6 nucleated spleen cells. The trypan blue test showed 85–90 per cent viable cells in the inoculum. In this and in all the following groups including the controls heparin was added so that 0.5 ml contained 10 I.U.

Group II. 15 mice received intravenously 0.5 ml *whole homogenate* (that is "crude semisoluble extract + "DNA protein") corresponding to an initial wet weight of 100 mg pooled donor spleen. 0.5 ml of this inoculum contained 4.5–5.5 mg dry material.

Group III 14 mice were given intravenously 0 5 ml suspension containing "nuclear fraction" ("DNA protein") corresponding to an initial wet weight of 100 mg pooled donor spleen

Group IV 14 mice received intravenously 0 5 ml of the suspension containing the cytoplasmic fraction ("crude semisoluble extract") corresponding to an initial wet weight of 100 mg pooled donor spleen

Within 2 hours after transfer each mouse in every group received the initial dose of 0 05 mg nitrogen mustard subcutaneously (Erasol IDO®) The nitrogen mustard treatment was repeated after 48 hours 4 days after transfer all mice were killed by ether inhalation In the meantime no mortality occurred

Control Groups

Donor mice 20 normal, randomly selected mice were killed and their spleens pooled in cold isotonic saline Spleen cell suspensions and extracts were prepared exactly in the manner described above

Recipients

Group V (corresponding to the experimental group I) 8 mice were given intravenously 0 5 ml of a suspension of spleen cells from normal untreated syngeneic donors This dose corresponded to 100 mg initial wet weight of pooled donor spleen and to approx 100 × 10³ nucleated cells Viability was 85 per cent

Group VI (corresponding to the experimental group III) 10 mice received intravenously 0 5 ml of a suspension of "DNA protein" corresponding to an initial wet weight of 100 mg pooled normal donor spleen

In the controls treatment with nitrogen mustard and time of killing were identical with those of the experimental groups No spontaneous mortality was observed.

An additional experiment under similar conditions (Experiment B) was designed in order to investigate 1) the histological events taking place during the first few days after transfer of "DNA protein" derived from spleens of casein treated donors with and without subsequent treatment of the recipients with nitrogen mustard 2) whether amyloidosis similarly could be induced by *subcutaneous* administration of "DNA protein" from casein treated donors and 3) whether the observed amyloid inducing potency of the "DNA protein" from casein sensitized donors might be influenced or even abolished by treatment of the inoculum with DNAse prior to injection

Experiment B

In Experiment B inbred randomized C3H mice of the same stock as those employed in Experiment A were used The treatment of the donors and the preparation and the fractionation of spleen cell extracts were identical to the procedure described in Experiment A

After 17 days of casein treatment spleens from 25 randomly sele[illegible] were harvested and pooled The "DNA protein" derived from this pool wa[illegible] with isotonic saline to a suspension containing the equivalent of 10[illegible] wet weight of pooled donor spleen per 0 5 ml 28 mice each received 0 5 ml [illegible] intravenously in the tail 14 of these mice (groups I–3 (T[illegible]) [illegible] subsequently treated with nitrogen mustard as described above and [illegible] 1 2 and 4 days after transfer The remaining 14 mice comprising th[illegible] were treated likewise except that they did *not receive* nit[illegible] (Table 1) 8 mice each were given 0 5 ml of the same "DNA [illegible] subcutaneously in the back followed by nitrogen mustard tre[illegible] they were killed after 4 days

An amount of the "DNA protein" equivalating 600 mg [illegible] of pooled donor spleen was washed and resuspended in 0 02 M phosphate [illegible] H 6 5) with 0 001

TABLE 1

Experiment 1. Number of animals developing amyloidosis and their mean degrees of spleen amyloidosis 4 days after the transfusion of whole viable spleen cells or various fractions of spleen cells from either normal mice or mice previously treated with sodium caseinate for 17 days

Group	Pretreatment of donors	Number of recipients in group	Treatment of recipients: Transfused material	Treatment of recipients: Nitrogen mustard injections (0.05 mg)	Recipients killed after	Number of recipients developing amyloidosis (of total)	Mean degree of recipient spleen amyloidosis
I	17 daily injections of sodium caseinate	12	Whole viable spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	12/12	+ + + (2–4)
II		15	Whole homogenate of spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	15/15	+ + + (2–4)
III		14	Nuclear fraction ("DNA protein") of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	14/14	+ + + (2–4)
IV	"	14	Cytoplasmic fraction ("crude semisoluble extract") of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/14	0 (0–0)
V	None	8	Whole viable spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/8	0 (0–0)
VI	"	10	Nuclear fraction ("DNA protein") of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/10	0 (0–0)

Experiment B Number of animals d velop ng amyloid si an l their mean degree of pleen amylol los s 1 2 or 4 days after the trans fusi n intravenously or sub utaneously of nucle r fr ction (DNA protein) deri ed fr m either normal mou e pleen cells or spleen cells from donors previou ly treate l vith s lium cas inate for 17 days

1	17 daily injections of o lium caseinate	5	Nuclear fraction (DNA pro tein) of sp leen cell homoge nate i v (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Day 1	24 hour	0/5	0 (0–0)
2		5		Day 1	? days	0/5	0 (0–0)
3		4		Days 1 and 3	4 days	4/4	+ + (2–3)
4		5		None	24 hours	0/5	0 (0–0)
5		4			2 days	1/4	(+) (0–?)
6		5			4 days	2/5	(+) (0–2)
7		8	Nuclear fraction (DNA pro tein) of spleen cell homoge nate *subcutaneously* (dose = the equivalent of 10 mg wet weight of pooled donor pleen)	Days 1 and 3	4 days	1/8	(0) (0–?)
8		6	DNA e treated nuclear fraction (DNA protein) of spleen cell homogenate i v dose = the equivalent of 100 mg wet weight of pooled donor spleen	Days 1 and 3	4 days	6/6	+ + (2–3)
9	None	11	Nuclear fraction (DNA pro tein) of spleen cell homoge nate i v (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/11	0 (0–0)

M MgCl so that 0 5 ml of the suspension contained DNA protein equivalating 100 mg wet weight of pooled donor spleen This suspension was thereafter incubated for 2 hours at 20 C with 40 microgramme of beef pancreas DNAse D6CA (Worthington) After incubation 0 5 ml was injected intravenously into each of 6 recipients which subsequently received the usual course of nitrogen mustard until killed after 4 days They comprised the *group 8* (Table 1)

Finally *group 9* comprised the controls corresponding to group VI of Experiment A (Table 1) 11 mice received DNA protein intravenously from normal untreated donor spleens derived from 15 randomly selected donors (dose = the equivalent of 100 mg wet weight of pooled normal donor pleen) After the usual course of nitrogen mustard the recipients were killed after 4 days

Histology

Tissues were fixed in neutral formalin paraffin embedded and sections were cut 5 microns thick The following stains were employed Haematoxylin eo in the P A S. technique the Feulgen stain and alkaline Congo red Amyloid was identified by morphology and bifringence with Congo red under crossed polars The individual degree of amyloidosis wa estimated semiquantitatively on sections of spleen and graded from 0–6 according to *Christensen & Hjort* (1959) In Table 1 mean values are expressed as plusses

RESULTS

The main results of the present experiments are outlined in Table 1 It will be seen that no amyloid at all could be found in mice receiving viable spleen cells or DNA protein derived from spleens of normal untreated donors (Experiment A groups V and VI Experiment B group 9)—a total of 29 recipients In contrast viable spleen cells whole spleen homogenate and DNA protein of spleen cells derived from casein treated donors were equally effective in inducing amyloidosis in the respective recipients while mice receiving crude semisoluble extract from the same pool of casein treated donors failed 100 per cent to do so Moreover in Experiment A the amyloid developing as a result of the various treatments apart from a 100 per cent positivity showed a striking degree of uniformity in the 3 groups receiving viable spleen cells whole spleen extract or DNA protein respectively (Figs 1–3) This uniformity points towards the DNA protein fraction as the agent responsible for amyloid induction in the recipients

From the results of Experiment B (Table 1) it appears that animals receiving DNA protein from casein treated donors did not develop recognizable amounts of splenic amyloid before at least 2 days after the transfer (groups 1–6) While in group 3 4 out of 4 recipients of DNA protein subsequently treated with nitrogen mustard developed amyloidosis when killed after 4 days only 2 out of 5 recipients in group 6 *not receiving nitrogen mustard* did so when similarly killed after 4 days This indicates that although nitrogen mustard markedly enhances amyloid formation in the recipients it is not completely necessary for the initiation of the amyloidogenic process in this system

From the observations in group 9 (Table 1) it is apparent that subcutaneous administration of the DNA protein to recipients subsequently treated with nitrogen mustard was nearly ineffective in inducing



Fig 1 Section of spleen from mouse treated with an intravenous injection of viable spleen cells from casein treated syngeneic donors The injection was followed by a short course of nitrogen mustard and the recipient was killed 4 days after cell transfer (experiment A group I) Spleen amyloidosis grade 3 Haematoxylin eosin × 35

Fig 2 Section of spleen from mouse treated with an amount of whole spleen homogenate from casein treated syngeneic donors equivalating 100 mg pooled wet weight of donor spleen Killed after 4 days of nitrogen mustard (Experiment A group II) Showing spleen amyloidosis grade 3 Haematoxylin eosin × 35

amyloidosis Only 1 out of 8 such treated mice responded with amyloid formation and only to a very slight degree

Finally all mice injected intravenously with DNAse treated DNA protein from casein treated donors followed by the usual course of nitrogen mustard (group 8) developed splenic amyloid to exactly the same degree as did mice similarly treated with intact DNA protein from the same pool

Morphologically the amyloid deposits were identical to those usually seen in experimental mouse amyloidosis They were predominantly arranged as perifollicular rings in the spleens The amyloid substance was moderately eosinophilic and weakly P A S positive except for the most peripheral parts where new formed amyloid showed a vivid purple —often in close relationship to P A S positive reticulo endothelial cells The amyloid substance stained pink with alkaline Congo red and exhibited the characteristic green birefringence when viewed under crossed polars In all sections examined the amyloid substance was Feulgen negative whole amyloid deposition in livers and kidneys was sparse or th



Fig 3 Spleen from mouse similarly treated with nuclear fraction ("DNA protein") from the same pool of casein treated syngeneic donors Nitrogen mustard and time of killing as above Spleen amyloidosis grade 3 (Experiment A group III) Haematoxylin eosin × 35

Fig 4 Spleen from mouse similarly treated with cytoplasmic fraction ("crude semi soluble extract") from the same pool of casein treated syngeneic donor spleens Administration nitrogen mustard treatment and time of killing as above (Experiment A group IV) No detectable amyloid resulted from this treatment Haematoxylin eosin × 35

DISCUSSION

That the transfusion of syngeneic spleens or their homogenates *per se* are not responsible for the initiation of the very rapid amyloidogenic process in the recipients of our transfer system seems evident from the negative results obtained with spleens from untreated donors in the present and in earlier experiments (*Werdelin & Ranlov* 1966) Thus it seems that only spleen cells or subcellular fractions of spleens from *hyperimmunized* donor mice are capable of initiating the rapid amyloidogenic process in syngeneic recipients Furthermore immunization (casein treatment) of the donor mice prior to cell transfer must endure at least 2 weeks of daily antigenic treatment (*Werdelin & Ranlov* 1966) The necessity of these conditions must be emphasized owing to the not infrequent findings of splenic hyalinization in such conditions as wasting disease or homologous disease attributable to graft *versus* host reactions in mice In these conditions the injections of immunologically competent allogeneic spleen cells into immunologically incompetent weanling F_1 hybrid mice may be followed 2–4 weeks later by a fatal wasting disease Among other pathological features this syndrome may

include the deposition in the splenic perifollicular regions of a homogeneous eosinophilic material which undoubtedly is amyloid (*Fiscus et al* 1962) In such conditions where the recipient F_1 hybrid spleen is populated by antigenically different parental strain cells the development of amyloid deposits in the course of a few weeks is to be expected in most strains of mice *Barnes et al* (1962) in lethally irradiated mice *inadequately* restored with syngeneic liver (fetal) or bone marrow described the development of secondary disease characterized by lymphoid aplasia hyperplastic granulopoiesis and from day 40 onwards widespread replacement of splenic tissue with an eosinophilic substance later recognized as amyloid (*Bradbury & Micklem* 1965) Provided an *adequate* protection with larger amounts of syngeneic lymphoid or bone marrow cells both the syndrome and the amyloid lesions were prevented Undoubtedly these irradiated and insufficiently restored mice were suffering from a variety of infections which alone will account for the subsequent amyloidosis These investigations too argue against any possibility of spleen cells *per se* being amyloidogenic when injected into adult *syngeneic* recipients This is further supported by *in vitro* studies by *Dutton* (1965) who showed little or no proliferative response occurring in mixed cultures of syngeneic spleen cells from mice In contrast a rapid increase occurred in allogeneic combinations

In the present experiments only nuclear fractions were capable of initiating the amyloidogenic process in the recipients As all of the *antigenic activity of cells has been found to reside exclusively in the* cytoplasmic fractions (*Medawar* 1963 *Monaco et al* 1965) there seems to be no reason to ascribe the amyloid inducing effect of our transfused material to antigenic properties—disregarding the syngeneic donor-host relationship

From our results it further appeared that recipients subsequently treated with nitrogen mustard developed amyloidosis far more markedly than did untreated recipients of the same inoculum Such an amyloid enhancing effect of nitrogen mustard was originally described by *Teilum* (1954) however the exact nature of the intracellular events induced by the mustard are not well understood

Among the effects of the various alkylating agents is an initial profound depression of the rate of incorporation of H^3 thymidine into DNA (*Wheeler* 196[illegible] *Booth et al* 1964) A parallel depression in the RNA and protein synth[illegible] though usually to a lesser extent (*Bifcoet & Busch* 1961 *Honig et* [illegible] 61 [illegible] *th et al* 1964) From 36 to 48 hours after the administration of the [illegible] in experiments employing normal murine as well as neoplast[illegible] in DNA content and large accretions of RNA and pro[illegible] al 1961 *Levis et al* 1963 *Booth et al* 1964) This accur[illegible] results in a pronounced swelling of the cells to a volum[illegible] an that of untreated cells (*Booth et al* 1964) The mechani[illegible] death is not clear Treatment of cells grown *in vitro* with a [illegible] e dose of nitrogen mustard does not produce any measurabl[illegible] physical or chemical properties of their DNA while DNA whi[illegible] with high concentrations of mustard [illegible] many physicochemical [illegible] among which a markedly reduced visco[illegible] (*Brewer & Aronow* 1963) [illegible] investigations

demonstrated the retention by DNA of increased amounts of protein unseparable by normal purification procedures (*Colder et al* 1964)

Recent experiments by *Chanmougan & Schwart* (1966) showed that increased as well as inhibited antibody formation may follow treatment of rabbits with 6-mer captopurine They proposed that nucleic acids released from the injured cells could account for the enhancement of the immune response in the former case

The constant association of lymphoid iplasia with the various conditions in mice characterized by wasting although bacterial or viral infections may account for many of their features has occasionally revived interest in a possible trophic function of lymphoid cells (*Loutit* 1962 *Barnes et al* 1962) As the possible mediators of such activity have been specified DNA or its break down products It has been shown that radioactively labelled DNA can be reutilized as such by other dividing cells in the animal for example by regenerating liver cells (*Bryant* 1962) or in skin grafts (*Diderholm et al* 1962) Polynucleotides may be incorporated directly into lymphocytes (*Hill & Pospisil* 1960) and strain L cells (*Bensch & King* 1961) The surprisingly long life span of lymphocytes as measured by their turn over of P labelled DNA (*Ottesen* 1954) has been interpreted as the reutilization of large fractions of the nucleic acids or nucleoproteins of their predecessors (*Hamilton* 1958 *Sundberg* 1960) *Trowell* s (1957) observation of pyknotic remains of phagocytized small lymphocytes within reticular cells made him submit a hypothesis of reutilization in a lymphocyte macrophage cycle (*Trowell* 1958) and modified versions of these views have later been expressed (*Sundberg* 1960 *Berman* 1966) Such a mechanism has been claimed to account for the transfer of immune reactivity by lymphoid cells between syngeneic individuals as being a transfer of self replicating nucleoprotein subsequently being reutilized as viable templates for antibody and/or protein synthesis (*Hamilton* 1958) Similarly a possible transfer of immune reactivity with nucleic acids or nucleotides between cells within the same individual has been proposed (*Chanmougan & Schwartz* 1966) However evidence seems largely circumstantial

On the other hand purified preparations of DNA have been shown to be capable of transferring murine leukemia (*Hays et al* 1957) and various tumours (*Laterjet et al* 1958) DNA from adult mouse L cells has been found highly stimulatory of *in vitro* polypeptide synthesis in contrast DNA from mouse embryos or peritoneal tumour cells were nearly inactive (*Holland & McCarthy* 1964) Nucleic acids and enzymatically digested nucleic acids have been shown capable of enhancing antibody synthesis (*Johnson et al* 1965 *Braun et al* 1965) An extensive review of problems concerning the possibility of nucleic acid mediated genetic transformation has recently been published by *Ledoux* (1965) The bearing of these observations on the results of the present experiments rely mainly on the fact that the material with which our amyloidosis apparently was transferred contained large amounts of nucleoproteins—that is DNA or DNAse treated DNA

The transferred agent which in the present material seems responsible for the initiation of the amyloidogenic process in the recipients is not likely to be identical with the transfer factor originally described by *Lawrence* (1960) This factor which hitherto has been demonstrated in humans only is capable of transferring delayed hypersensitivity to normal recipients Transfer factor itself appears to be a low molecular weight dialysable material (*Lawrence et al* 1962) it has been identified in a polynucleotide fraction and in a fraction containing various globulins and RNA both fractions derived from human white blood cells (*Baram et al* 1966) The search for such a transfer factor in animal species has as a whole not been successful Assuming that such a factor akin to the transfer factor of Lawrence s was operating in the present material it should be present in the supernatant fraction of the spleen cell homogenate which fraction on the contrary failed to induce amyloidosis in our recipient mice

It is only reasonable to believe that the particles of our "DNA protein suspension when injected intravenously primarily ended up in phagocytizing cells of the recipients reticulo endothelial systems preferably in the spleens and livers which are known to be the primary sites of murine amyloid formation Moreover this will account for the failure to induce amyloidosis with the same inoculum when administered subcutaneously

The direct demonstration of the participation of reticulo endothelial cells in amyloid formation has been repeatedly demonstrated (*Teilum* 1954 1956 1964 *Cohen et al* 1965) From these and other investigations (*Werdelin & Ranlov* 1966 *Ranlov & Werdelin* 1967) it seems indisputable that the amyloidogenic process is mediated primarily by *cells* thus ruling out any speculations concerning circulating precursors etc The two-phase cellular theory of local secretion (*Teilum* 1964) clearly points out the existence of two consecutive events in the mesenchyme 1) A primary preamyloid phase of weeks duration characterized by increasing cytoplasmic pyroninophilia of reticular and plasmacytoid cell lines 2) In the course of persistent antigenic stimulation the second amyloid phase gradually sets in characterized by the accumulation at the same sites of amyloid deposits in the immediate vicinity of large often P A S positive reticulo endothelial cells with the onset of the second amyloid phase the initial pyroninophilia gradually disappears However the relationship between the pyroninophilic cell of the initial phase and the P A S positive reticulo endothelial cell o the se ond phase undoubtedly synthesizing amyloid has nev been clarifi d Are they identical or at least do they represent the sam cell l n th latter being the result of differentiation of the former? In our e [illegible] e peri ments in which pyroninophilic spleen cells from casein re d donors caused amyloid to develop in syngeneic recipients [illegible] fel justified in assuming that this was the case (*Werdelin & Ranlov* 1966 *Ranlov & Werdelin* 1967) However the results of the present experiments do

indicate their relationship as an indirect one involving an intermediate stage of cell death or cell fragmentation On this account and within the limits of the biphasic cellular theory the following mechanism of experimental amyloid formation may be suggested

1) During prolonged antigenic treatment an increasing stimulation of mainly cellular immune reactions occurs (*Ranlov* 1967 *Ranlov & Jensen* 1966) This hyperstimulation leads to increasing pyroninophilia of various reticular and plasmacytoid cells —the former probably representing the activated state (blast transformation) of lymphoid cells corresponding to the simultaneous disappearance of small lymphocytes in the perifollicular regions of the spleen The pyroninophilia reflects the rapid increase in the production of various antibodies nucleic acids proteins and enzymes necessary for cell division

2) After a few weeks of persistent antigenic stimulation the capacity for further proliferation is used up and a state of exhaustion is reached From now on the pyroninophilia will decrease and on the cellular level the dominating feature will be cell death or cell fragmentation This is an intermediate stage and represents the so called inversion phase (*Teilum* 1964)

3) The increasing cell death (or cell fragmentation) results in the release of various nuclear and cytoplasmic fragments some of them self replicable nucleic acids or nucleotides carrying some kind of information from their metabolically highly active parental cells These fragments of nuclear material are locally phagocytized in R E S cells Under the specific conditions of over loading the ingested (self replicable) material will impose protein synthetizing information on the R E S cell thus causing a profound disturbance in its function This disturbance may either be a direct one the information being imposed directly on the phagocytizing cell itself or it may be an indirect one the information being passed on to other reticular cells (nursing) with different potencies The distinction between these possibilities on histological grounds is not facilitated in the light of evidence of the labile nature of the morphologic appearance of the various cells that are involved (*Berman* 1966) The visible results will be a cytoplasmic P A S positivity of reticular cells indicating intracellular synthesis of glycoproteins and extracellular deposition of amyloid

SUMMARY

The adoptive transfer of experimentally induced amyloidosis between syngeneic mice is described It appears that not only viable spleen cells but also the nuclear fraction of spleen cell homogenates derived from casein treated donors are effective in initiating a rapid amyloidogenic process in healthy recipients when administered intravenously while cytoplasmic fractions are not Only spleens from donors presensitized with casein were capable in these respects

Of a total of 51 mice receiving viable spleen cells whole spleen extract nuclear fraction of spleen cells and DNAse treated nuclear fraction respectively derived from casein treated donors all 51 mice developed amyloidosis to the same degree within 4 days while 14 mice receiving cytoplasmic spleen cell fraction derived from the same casein treated donors all were negative as were 7 out of 8 recipients treated with the "nuclear fraction subcutaneously

29 control mice receiving corresponding doses of spleen cells or spleen extracts from normal non sensitized donors failed 100 per cent to develop amyloid lesions In all cases including the controls a short course of nitrogen mustard treatment was given after the transfer

From other similarly treated groups subjected to serial killings after 1 2 and 4 days it appeared that amyloid did not develop in the recipients until 48 hours after the transfer

The results are taken as supporting a possibility of different cell types operating specifically for each of the two phases in experimental amyloid formation

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DNA SYNTHESIS IN FREE FULL SKIN AUTOGRAFTS IN MICE

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Received 23 xi 66

The take of a free skin autograft is comparable in many aspects to the closure of a surface wound by apposition of the skin edges The epidermal mitotic activity plays a dominant part in the healing of the wound (*Montagna* 1962)

It has been established that free skin autografts get initial blood circulation at the time when the grafts loose their edema *e g* on the 4th or the 5th day after grafting (*Clemmesen* 1965) Other observations reveal that free skin autografts have a reduced blood circulation during the first 6 days after the grafting procedure and the blood circulation was determined active or normal on the 7th day (*Marcl mann* 1965)

Once the blood circulation is established in skin grafts it should be possible to demonstrate epidermal deoxiribonucleic (DNA) synthesis in dividing cells with tritiated thymidine distributed by the circulation which has been demonstrated to be a reliable method of labelling dividing nuclei (*Messier & Leblond* 1960) We thought it might be of interest to evaluate the regeneration of the epidermal layer in skin autografts in accordance to incorporated tritiated thymidine during the initial phase of the healing process after grafting

MATERIAL AND METHODS

Sixtyone female mice of a non homologous strain 4 weeks old and weighing about 20 to 30 grams each were used as experimental animals The investigations were performed in two experimental groups of which the first was a preliminary

A modified technique of free skin autografting in mice described earlier (*Cross et al* 1960) was used Nembutal was administered intraperitoneally in a dose 0 06 mg per 10 grams of body weight The mice were shaved dorsally After application of an adhesive film onto the grafting area a 5 cm strip of surgical micropore tape was applied across the dorsum just anterior to the femoral insertion of the hind legs A 1 × 1 cm piece of full skin was cut and the axis of the graft was turned 180 degrees in regard to the bed The graft was again fixed to the bed with surgical micropore tape

An hour before killing the animals *2 µc per gram of body weight of tritiated thymidine*[1] were injected intraperitoneally

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[1] Schwarz BioResearch NC Orangeburg N Y Sp Act 3 0 c mm le



Fig 1

Completely necrotic transplantate 5 days after transplantation Magnification 6×10

In the pilot experiment the mice were killed 4 5 and 6 days after skin grafting and in the second group 4 5 6 7 9 10 11 and 12 days subsequently

Histologic specimens were cut from the middle portion of the grafted area including the host—graft junctions on both sides of the graft as well as normal skin outside the grafted area The histologic samples were fixed in 4 per cent formalin and cut into 4 μ sections

Autoradiograms were prepared by the dipping method with Ilford K 5 photoemulsion and 10 days exposure time was used After development the preparations were stained with haematoxylin eosin

Transplantates in the tables have been divided in 3 groups

a) completely necrotic

b) partly regenerated (regeneration foci in the middle or in the border areas of necrotic epidermis)

c) completely regenerated (epidermis normal throughout the whole area of the transplantate)

Incorporation of ^{3}H thymidine in the premitotic cells has been used as regeneration indicator Cells were considered labelled with grain count at least 5 times above the background

RESULTS

Experiment 1

This experiment was performed to find a good grafting technique and the best postoperative period for investigating epidermal DNA synthesis in the grafted skin

The experimental series included 14 mice Tab 1 hows that the epidermal layer of the grafted area h d par [illegible] y [illegible] erated (= in accordance with incorporated tritiated th mi [illegible] [illegible] onl one animal



Fig 2

Complete y regenerated transplantate 10 days after transplantation
Magnification 6 × 10

4 and 5 days after grafting. In animals sacrified 6 days after grafting a partial regeneration of the epidermal layer was noticed in all test animals

TABLE 1

Regeneration of the Epidermal Layer in Free Full Skin Autografts in Accordance with Incorporated Tritiated Thymidine

Time after transplantation	Number of animals	No regeneration (completely necrotic)	Regeneration Partial	Regeneration Complete
4 days	7	6	1	0
5 –	3	2	1	0
6 –	4	0	4	0

Experiment 2

In this series the experiments in the first group were repeated and continued This group included 47 test animals The results are shown in Table 2

An increased number of labelled cells were seen in grafts 7 days-old indicating epidermal regeneration The epidermis was almost completely regenerated from the 10th day on after the grafting procedure and the regenerative process continued in an increasing manner 12 days after the grafting

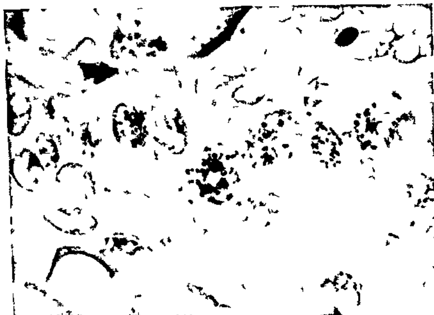


Fig 3
Completely regenerated transplantate 10 days after transplantation
Magnification 6 × 100

TABLE 2
Regeneration of the Epidermal Layer in Free Full Skin Autografts in Accordance with Incorporated Tritiated Thymidine

Time after transplantation	Number of animals	No regeneration (completely necrotic)	Regeneration Partial	Regeneration Complete
4 days	5	4	1	0
5 –	8	5	3	0
6 –	2	1	1	0
7 –	5	1	4	0
9 –	7	4	1	2
10 –	7	1	3	3
11 –	7	0	4	3
12 –	6	1	1	4

There were more activity in the edges of the grafts than in the central parts of the epidermal area. In some sections increased labelled DNA synthesis were noticed even in the central parts of the graft.

Vascular congestion and numerous leucocytes were seen in the dermis 4 to 6 days after grafting. Regenerating mesenchymal cells of the dermis were seen in the earlier groups before regeneration was observed in the epidermal layer.

DISCUSSION

The results reported reveal that increased epidermal DNA synthesis appears in cells of free full skin autografts in mice from the 7th post operative day increasing at least until the 12th postoperative day

These findings correlate well to earlier observations on the blood circulation in free skin autografts According to Marckmann (1965) the grafted skin has a reduced blood circulation during the first 6 days postoperatively whereas the circulation was considered active or normal on the 7th day There was no or very low activity in the epidermal layer during the 4 5 6th day although the skin graft should have established anastomosis between the vessels The first burst of mitotic activity appeared after the blood circulation can be considered normal (*Brophy & Lobitz* 1959 *Clemmesen* 1965 *Marckmann* 1965 *Pinkus* 1952)

We were not able however to determine the source of the labelled cells in the autografts whether they were cells of the grafts themselves or cells migrating from the epidermal layer outside the graft The experiments will be continued in this respect

SUMMARY

The study comprises a total of 61 female mice divided into two test groups An attempt was made to investigate the epidermal DNA synthesis in free full skin autografts with tritiated thymidine injected 4 5 6 7 9 10 11 and 12 days after the skin grafting procedure The results revealed that a definite increased regenerative activity of the epidermal layer of the grafts measured with incorporated tritiated thymidine occurs on the 7th and until at least the 12th day postoperatively The increased epidermal DNA synthesis correlates well to the established active blood flow that free skin autografts get on the 7th day after grafting

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ELECTRONIC CELL COUNTING IN CELL CULTURE STUDIES

By

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Received 30 xii 66

Counting of cultured cells by the haemocytometer technique is time consuming and the total error of the method has been estimated to some 9 5 per cent (*Biggs & MacMillan* 1948) Counting of erythrocytes by an electronic cell counter[1] has been studied by *Oulie* (1959) who found a mean error of 0 85 per cent due to the apparatus and a total mean error (including sampling) of 2 1 per cent These values correspond well to the results obtained for another similarly designed counter (*Brecher et al* 1956 *Mattern et al* 1957) Compared with the haemocytometer procedure the electronic counting thus provides more exact erythrocyte counts

In order to evaluate the applicability of electronic counting in cell cultivation Celloscope counts obtained from suspension cultures of P-388 cells have been analyzed

MATERIALS AND METHODS

Cell Cultures

The cultures employed in this study were all suspension cultures of P-388 cells (*Dawe & Potter* 1957) The growth medium was a slight modification of Eagle's Minimum Essential medium (*Eagle* 1959) Methocel (*Earle et al* 1954) was added to a final concentration of 1 [illegible] g/l and pluronic acid (*Swim & Parker* 1960) to 1 0 g/l Phenol red was used as a pH indicator and penicillin (100 I U /ml) and streptomycin (100 µg/ml) were added To the medium was added 10 per cent of heat inactivated horse serum

Counting Procedures

The celloscope has been constructed mainly for [illegible] The erythrocytes are suspended in salt solution and p[illegible] through [illegible] by suction At the same time an electric current is [illegible] through [illegible] The passage of each er[illegible] reduces the conducti[illegible] the sy[illegible] is proportion[illegible] volume The cell susp[illegible] single cells pass the capill[illegible] change in resistance [illegible] amplified [illegible]minator and is finally registered [illegible] an electronic coun[illegible] The disc[illegible]nes the minimum change of r[illegible]nce (or minimum [illegible] corded As

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[1] Cellos ope [illegible] & Co., Stockholm Sweden

all particles exceeding a certain size will be registered the diluting medium should be filtered and its background count determined

In the actual experiments a solution of 0 85 per cent NaCl was used Several 1 ml volumes of this solution were run through the apparatus preceeding each test to wash out cells and debris from previous tests The background count of the saline was 2 to 4 (i e 0 5 to 2 per cent of the total count) and the culture samples were prepared by adding 20 ml of saline to 1 ml of cell culture The dilution was prepared in an Erlenmeyer flask transferred to a small beaker and the counting performed immediately The preparation of the dilution would take 3 to 4 minutes For each sample the counts of three separate 1 ml volumes were registered and the cell concentration was calculated from the mean of these single counts The whole procedure would take some 10 minutes

The discrimination knob was used in position 40 According to the instruction leaflet which is concerned with erythrocyte counting this would exclude cells with a volume less than 68 μ^3 Assuming that the P-388 cells have a roughly spherical form this would correspond to P-388 cells with a diameter of approximately 5 μ

Visual Counting in the Haemocytometer

Care was taken to avoid uneven distribution of the cells due to settling Samples were taken with Pasteur pipettes and transferred undiluted to a Barker haemocytometer The cells in 5 A squares (each 10^{-4} ml^3) were counted and the highest and lowest values were disregarded The mean of the three remaining values was registered as the cell count

RESULTS

All experiments recorded except one were performed in 4 Erlenmeyer shake flasks Two flasks (A and B) had serum from lot I and showed actively proliferating cells that appeared normal under the microscope The two other flasks (C and D) were supplied with serum from lot II and contained cells which multiplied slowly and had a granular appearance Accumulation of debris was seen in the medium of these latter cultures

In experiment 3 samples were taken from a spinner flask The cells were multiplying at a fair rate and showed no morphological signs of degeneration

1 *Exactness of the Apparatus*

The mean error of Celloscope counts of erythrocytes has been found to be about one tenth of that observed with visual counting technique (*Oulie* 1959) The apparatus was tested for P-388 shake cultures in a similar way Cell count registration was performed as described and the discrepancies between individual counts were compared to the corresponding variations obtained with visual counting technique

The results indicate that within the same sample the *Celloscope* records values far more accurately (Table 1)

The relation to variations in individual visual counts is of the same magnitude as that reported for erythrocyte counting

With both methods there was a convincing trend towards a smaller variability with great cell density

TABLE 1

Celloscope and Haemocytometer Counts Variations within the same Sample

Sample No	Celloscope values Mean	Range	Range/mean	Haemocytometer values Mean	Range	Range/mean
1	255	7	1 37	24	7	14 6
2	264	6	1 13	34	7	10 3
3	265	5	0 94	34	11	16 ?
4	?64	1?	2 ?7	35	10	14 3
5	?97	3	0 50	32	6	9 4
6	309	6	0 97	3?	2	3 1
7	276	1	0 18	?9	9	15 5
8	?62	5	0 95	38	8	10 5
9	441	4	0 45	54	1?	11 1
10	438	6	0 68	56	2	1 8
11	418	3	0 35	49	3	3 0
1?	4??	4	0 48	46	1	1 1
13	650	8	0 61	104	13	6 3
14	705	8	0 56	113	11	4 9
15	571	4	0 35	71	6	4 ?
16	613	2	0 16	66	10	7 6
			x̄ 0 75			x̄ 8 37

P 388 cells cultivated in suspension were counted The Celloscope values are the numbers registered on the electronic counter the haemocytometer values express the cell density in the culture (cell 10⁴/ml)
Range presented as ± per cent of mean

2 *Evaluation of the Sampling Error*

Variations in the counts for one sample as well as between samples from the same culture have been recorded in Table 2 Although the variations between counts from different samples were on the whole greater they seemed to be within the range expected within a single sample

TABLE 2

Sampling Error of Celloscope Method

Variations within the sample Sample	Mean	Range	Range/mean	Variations within the culture Culture	Mean	Range	Range/mean
A1	334	2	0 3				
A2	3?5	2	0 4	A	?30	9	1 5
B1	340	2	0 3				
B?	3 5	?	0 4	B	331	13	2 1
C1	372	0	0 0				
C2	371	4	0 6	C	371		0 3
D1	363	?	0 3				
D?	3?1	?	0 3	D	36?	?	0 3
			x̄ 0 3				x̄ 1 0?

Variations in individual counts within a sample and between samples from the same culture of P 388 cells cultivated in suspension [illegible] A and B were well growing cultures C and D partly [illegible]
Range presented as ± per cent of mean

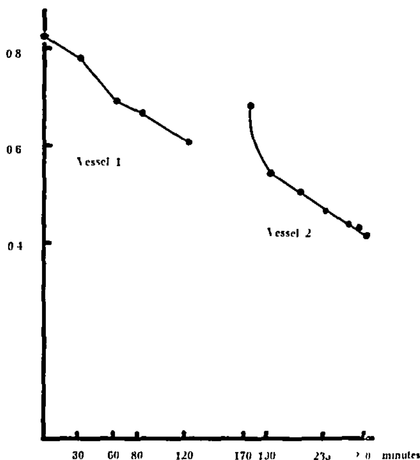


Fig. 1

Celloscope counts as a function of time interval from sampling and sample dilution to cell count registration. Two samples of 10 ml were harvested at t_0, diluted with 200 ml of 0.85 per cent filtered saline each and left at room temperature. Subsamples were counted at the times indicated on the figure. Preceeding each counting the vessel was agitated to avoid settling of the cells. Each plot represent the mean of three individual counts.

3. *The Effect of Time Interval from Sampling to Cell Count Registration*

To 10 ml samples from a spinner flask (t_0) was added 200 ml of filtered 0.85 per cent NaCl and the diluted samples were left at room temperature in Erlenmeyer flasks. Countings were performed at intervals through a period of 4½ hours. Preceeding each counting the Erlenmeyer flask was gently rotated to avoid uneven cell-distribution. Twenty ml of the dilution was then transferred to a small beaker and counting performed. Fig. 1 shows that the cell count decreases after sampling

and sample dilution The decrease starts immediately and is fairly proportional with time

4 *The Effect of the Nature of the Diluting Medium*

As the Celloscope counting procedure necessitates a dilution of the sample the effect of the diluting medium was tested The growth medium (containing 3 per cent of horse serum) and 0 85 per cent filtered saline were compared Each dilution was performed immediately before counting and cells from rapidly dividing and from more stationary cultures were counted after dilution in both liquids The results are presented in Table 3

TABLE 3

Effect of the Diluting Medium on Celloscope Counts

Sample source Flask	Counts obtained after dilution in Saline	Growth medium
A	473	486
B	464	504
C	495	451
D	488	472

P 388 cells cultivated in suspension were counted Cultures A and B were well growing cultures C and D partly degenerating One ml samples were diluted with 20 ml of the respective diluting media 0 85 per cent filtered NaCl and the growth medium (containing 3 per cent horse serum)

Mean of 3 individual counts The range within each sample was of the magnitude of 2 to 10

For both types of cultures the difference between parallel cultures was greater when growth medium was used for dilution A difference was observed between well growing and stationary cultures the former gave higher values in growth medium whereas the latter presented the higher values when saline was used as the diluting medium

5 *Haemocytometer and Celloscope Counts from Rapidly Dividing and more Stationary Cultures*

Two of the cultures tested showed signs of degenerat[illegible] wh[illegible]reas in the two others no such signs could be detected [illegible]ell coun[illegible] r[illegible] recorded for a period of four days After two days th[illegible] c[illegible]tures [illegible] n[illegible]nted with fresh medium corresponding to a [illegible]on of [illegible] F[illegible] 2 presents the cell inc[illegible]ease with time a[illegible] [illegible]mined [illegible] [illegible]ve counting methods

It is evident [illegible]hat [illegible]he Celloscope co[illegible]n[illegible]s and [illegible] [illegible]ytometer counts would lead to different conclusio[illegible] [illegible] th[illegible] [illegible] The Celloscope cannot reve[illegible] [illegible]ifference in grow[illegible] [illegible] [illegible]al technique a distinct differ[illegible] [illegible] found

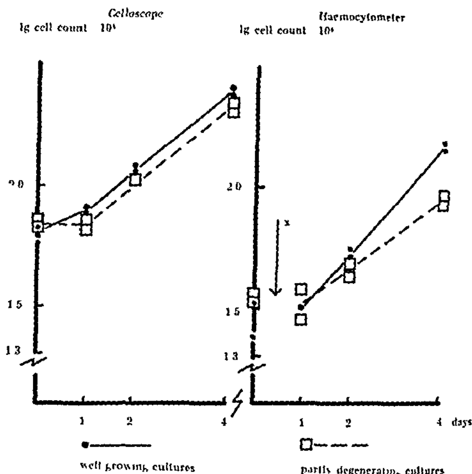


Fig 2

The effect of cell culture condition on Celloscope and haemocytometer counts. Two well growing and two partly degenerating shake flask cultures of 1 388 cells were *counted for a period of 4 days Each plot represents the mean of three individual* counts

X Variations between parallel samples too great to allow reasonable exactness of the curves

DISCUSSION

The present experiments apply to the reproducibility of the method as well as to technical procedures involved in preparation of the samples Lastly the specificity of the apparatus as regards particle nature was tested

For particle counting in general the Celloscope is a very precise instrument The results in Table 1 show that individual counts in the Celloscope vary far less than in the haemocytometer and that the Celloscope presents the greatest advantage when counting cultures with a low cell density The relative exactness of the methods corresponds well to the values reported for erythrocyte counting (*Ouhe 1959*)

The data recorded to estimate the error introduced by sampling (Table 2) are too few to give more than purely tentative informations. However the sampling error was found to be relatively small. The total error (including sampling error) with Celloscope technique was far less than the variations within the same sample in the haemocytometer (Tables 1 and 2). An additional advantage is obvious: the Celloscope method eliminates the personal bias involved in visual counting.

However the couting of cultured cells presents special problems. The experiment concerned with the effect of the diluting medium indicates that this factor is of some importance (Table 3). In what way this factor affects the results cannot be evaluated from the present experiment. Because the difference between parallel cultures was greater after dilution in growth medium, saline was chosen for further tests. The choice was further based upon the claim that fibrous material is considered to be an undesirable contamination (*Mattern et al* 1957).

The time necessary for sampling and sample dilution greatly influenced the cell counts which decreased proportionally with time. One value not in agreement with this statement (Fig 1 $t_{1\,8}$) was probably due to instability of the apparatus: the range of the individual counts around this value was 94, ten times the average range value in this experiment. It is evident that saline also influences the counts, possibly by causing cell disintegration, and the time factor must be strictly defined before conclusions are attempted.

Fig 2 shows that the Celloscope does not seclude particulate matter other than viable cells. With the present technique the Celloscope registers debris accumulation in the same manner as cell multiplication. This can also to some extent explain that the cell counts obtained with the Celloscope are higher than the visual counts from the same culture (Fig 2).

It must be concluded that for particle counting the Celloscope *per se* is a far more precise tool than the haemocytometer. However the counting of cells propagated in culture presents special problems. The influence of the growth medium and the additional counts from sources other than viable cells must be controlled before the Celloscope can replace the haemocytometer in cell culture studies.

SUMMARY

1. Cell counting in cell culture experiments has been performed. Conventional visual technique and an electronic counter (Celloscope 101) have been compared.
2. The Celloscope gave individual counts which varied far less than the individual counts in the haemocytometer.
3. The Celloscope counts were influenced by the diluting medium and by the time lapse from sample dilution to cell count registration. These factors should be standardized.

4 The Celloscope method did not discriminate satisfactorily between rapidly dividing and more stationary cultures Because of this fact the apparatus must be considered less suitable for cell culture studies until the method has been utterly refined

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A NEW TISSUE CULTURE METHOD FOR STUDIES ON ANTIBODY SYNTHESIS IN VITRO BY LYMPHOID CELL SUSPENSIONS

By

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Received 30 i 67

Tissue culture of lymphoid cells poses some special problems (cf *Trowell* 1965) It has none the less been extensively used for immunological studies (reviewed by *Stavitsky* 1961 *Amis* 1964 *Nossal* 1966) A continuous antibody production lasting for some time *in vitro* has been demonstrated repeatedly with the use of lymphoid cells stimulated *in vivo*

Lymphoid cells can be cultured separately as cell suspensions or in organ cultures as tissue fragments or slices The former method has some advantages as pointed out by *inter al* *Vaughan et al* (1960) *La Via* (1962) *Michaelides & Coons* (1963) *Halliday & Garvey* (1964) and *Svehag* (1965) Svehag stresses the following points a/ quantitative and qualitative uniformity of replicate cultures b/ uniformity of environment for all cells of a culture c/ uniform and rapid uptake of compounds incorporated into the medium d/ elimination of intercellularly retained antibody (p 261) On the other hand cell suspensions have usually resulted in less antibody production than organ cultures have (see however conflicting views by *La Via* 1962 *Dresser* 1965) Antibody production has usually been demonstrated for only a short time a few hours to a few days (see however e g *McKenna & Stevens* 1960 *Fuji & Prince* 1964) The highly sensitive methods needed for demonstrating antibodies may not only increase sources of error (cf *Stavitsky* 1961) but may also increase the difficulty in distinguishing between antibody synthesized *de novo* in the culture and antibody synthesized *in vivo* and released during cultivation (cf *Stavitsky* 1963) Only rarely has enough antibody production been obtained *in vitro* to permit its demonstration with immunoprecipitation a method by which some of the difficulties mentioned above could be avoided *Patterson et al* (1964) utilized the fact that the spleen in [illegible] can

This investigation was financed by grant [illegible] from the S[illegible] *[illegible]ut[illegible]pl[illegible] Sclerosis Foundation* from the *Swedish Medical Research [illegible]* [illegible] 579-0?) and from the *Swedish Cancer Society* (project 6[illegible])

produce large amounts of precipitating antibodies In some of their experiments an antibody production *in vitro* could be found of enough strength to permit immunoprecipitation

The aim of the present investigation was to find a simple method for cultivation of lymphoid cells in suspension for many days and under conditions that made antibody synthesis *in vitro* demonstrable with the use of precipitation methods The culture method should be simple and easy to modify for various experimental purposes The source of cells was rabbit lymphoid cells stimulated *in vivo* The presence of antibody containing cells was studied with immunofluorescence and compared with the presence of antibody in the culture media

MATERIAL AND METHODS

Antigen Commercial human γG globulin (AB Kabi Stockholm) was used as antigen for immunization A purer preparation (Kabi 1349) was used for immunofluorescence and immunodiffusion studies (For further details see Nilsson 1967)

Preparation of lymphoid cell suspensions A regional lymph node and/or the spleen was teased apart with two forceps in Parker 199 solution filtered through double gauze and washed by centrifugation three times with Parker 199 The number of mononuclear cells was determined and suspensions with cell concentrations between 10 and 120 mill/ml were prepared

Experimental groups Adult rabbits weight 2 3 kg mixed stock were used Four groups were studied

1) *Adjuvant treated controls* Three rabbits were injected twice intracutaneously ten days apart with 0 1 ml 0 9 per cent saline emulsified in the same volume of Freund s complete adjuvant (Difco) Cell suspensions were prepared from regional lymph nodes and spleens four days after the second injection

2) *Intravenously and intraperitoneally immunized rabbits* Two rabbits were injected with human gamma globulin intravenously (20 mg per dose) and intraperitoneally (60 mg per dose) for one month according to the schedule suggested by *Boyd* (1956) One day and two days respectively after the last intravenous injection cell suspensions were prepared from the spleens On this occasion immunodiffusion revealed antibodies in both rabbit sera whereas Coombs test revealed antibodies in only one (titer 1 4000)

3) *Intracutaneously immunized rabbits heterogenous group (immunization schedule varied)* Seven rabbits were injected intracutaneously with a volume of 0 1 ml of human gamma globulin emulsified with 0 1 ml Freund s complete adjuvant The antigen dose varied from 1 to 48 mg Two to five injections were given during ½–4½ months Cell suspensions were prepared two three or four days after the last injection Both immunodiffusion and Coombs test (titers between 1 256 and 1 130000) revealed antibody in serum Separate cell suspensions were prepared from the regional lymph node and from the spleen in two rabbits In each of the other five rabbits cells from the lymph node and the spleen were mixed Thus nine cell preparations were studied from the seven rabbits

4) *Intracutaneously immunized rabbits homogeneous group (immunization schedule standardized)* Eight rabbits were given two injections ten days apart each injection contained 6 mg human gamma globulin in a volume of 0 1 ml emulsified with 0 1 ml Freund s complete adjuvant Cell suspensions were prepared from a mixture of regional lymph node and spleen four days after the last injection Immunodiffusion and Coombs test (titers between 1 128 and 1 51[illegible]) revealed antibody in serum

Culture method Cells were suspended in a medium composed of 70 per cent Parker 199 and 30 per cent autologous serum taken before the first injection Antibiotics were added to the medium 50 I U of penicillin and 50 γ of streptomycin per ml This medium is in the following referred to as the *culture medium* in contrast to the *depot medium* described below Fig 1 shows the culture arrangement One or more dialysis tubings [illegible] cm diameter (Union Carbide Corp Chicago Ill) are

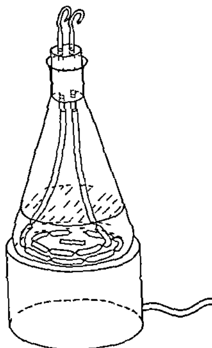
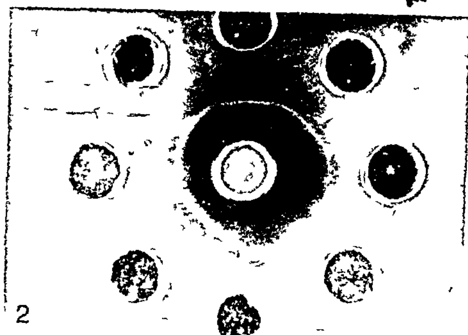


Fig 1
Drawing of the culture arrangement For explanation see text

connected with glass tubes that perforate the stopper of a 500 ml Erlenmeyer flask The cell suspension is injected into the tubing through a thin rubber tube connected to the external end of the glass tube In order to distribute the cells along the dialysis tubing it is divided after filling into short segments with perlon thread The tubing can be divided into separate compartments by tying the tubing itself into knots or separate tubings can be tied together after filling These steps are performed before the tubing is placed in the flask The compartments can then be removed separately during cultivation The flask contains a depot medium (175 ml Parker 199 75 ml horse serum and antibiotics in the concentration mentioned) kept in circulation by a magnetic stirrer In the present study there was no need to renew the depot medium The flask is placed in a thermostat at 37 C Up to three separate dialysis tubings each 90 cm long have been placed in the same flask

After the end of the culture period the cells were washed three times in saline and used for immunofluorescence studies whereas the culture medium was always concentrated 10–20 times in a collodion bag (*Mies* 1953) before being analysed for antibodies

Studies on cell survival during cultivation Three rabbits were injected intracutaneously with human gamma globulin emulsified with Freund s complete adjuvant according to the schedule of the homogenous group mentioned Antibodies in sera from these rabbits could be detected by both immunodiffusion and Coomb s test (titers between 1 16 and 1 1 8) four days after the second injection of antigen when cell suspensions were prepared from the regional lymph nodes and the spleen These rabbits were exclusively used for *studies on cell survival* Suspensions with 60 mill cells/ml culture medium were cultured as shown schematically in Fig 1 for four eight and 12 days At the start of the experiment and after each culture period cells from each preparation were cultured for six hours in test tubes with a cell concentration of 2 mill ml and dl valine H3 (The Radiochemical Centre Amersham England Specific activity 480 mC/mM) was added to the culture medium in an amount of 25 *µC/ml culture medium* One part of the cell suspension was





Figs ? 3

Fig ? Immunrdiffusion in agar Un tained slide In the c t l well wa placed concentrated culture medium in the peripheral well dilutions f the antigen human gamma globulin in diluti n tej f 1

Fig 3 Immun diffusi n in agar —reacti n of identity Un t l lik In the l wer well is the antigen human gamma gl bulin In the u r wells are immune serum and concentrated culture medium resp u ins terial from the same rabbit



Fig 4

Immunoelectrophoresis Unstained slide In the middle well is concentrated culture medium in the side troughs the antigen human gamma globulin Distinct lines at the site of γG

stained with acetic o cein after fixation for a few minutes in 60 per cent acetic acid The total number of cells per ml culture medium and the proportion of cells that in the orcein stained preparations looked healthy were determined at each occasion Another part was used for autoradiography The cells were air dried and fixed with two per cent neutral formol and carefully rinsed Autoradiography was performed with stripping film method using Kodak AR 10 plates and an exposure time of two weeks The cells were stained through th film with hematoxylin

Coombs test Human O Rh+ erythrocytes were coated with anti D serum Uncoated erythrocytes were used as controls Sera were diluted in serial 2 fold steps in saline Culture media concentrates were first diluted 20 times and then further diluted as ab ve

Immunofluorescence method Immunofluorescence vas carried out by an indirect method For further details see *Nilsson* (1967) In the first step human gamma globulin was used preincubated with a pool of normal rabbit serum In the second step fluorescein isothiocyanate (FITC) conjugated goat antiserum globulin vs human serum globulin (Microbiol Assoc Bethesda Maryland Control 49465) preincubated with the same rabbit se um pool was used I reincubation was carried out to eliminate non specific fluores ence (*Nilsson* 1967)

Two types of cont ol slides were made In t pe one only normal rabbit serum pool was used in the first step the second step being as above In type two the first step was unchanged but in the second step preincubated FITC-conjugated goat antiserum globulin vs guinea pig serum globulin (Mi robiol A soc Control 44315) was used

Immunodiffusion in agar gel Serum taken from each rabbit before and after injection schedule and oncentrated culture medium from each experiment were studied with Ouchterlony s m cro method with a standard equipme t (LI B Stockholm) 15 µl of the serum or of concentrated m dium was placed in the central well and 5 µl of the antigen human gamma globulin in the peripheral wells A wid ang of antigen dilutions was used Fig ? shows an analysis of a con ent t d ultur medium

For verifying the identity between antibod in the culture med um and th erum from each rabbit the follo ving investigation was made An antigen corc ntr t n was determined wh ch gave a sharp precipitate both with the con er t te med um and with the immune serum Antigen in this concentration was pl ced the c ntral well the concentrated culture medium and the immune serum wer pls a n adj cent pe ipheral wells When the precipitates were confluent as Fo a F 3 it was regarded as a sign of identity between the ant bod in the cu u e me n and that in the erum

Immun electrophoresis was perfo med according t S he gg r m r m thod 10 µl of serum or concentrated culture medium was placed i the m dd e w l After c mpleted electroph resis 50 µl of the antigen human gamma gl bul r was added

in the side troughs Fig 4 shows an immunoelectrophoresis of a concentrated culture medium

Studies on antibody synthesis The antibody content in the cells of regional lymph node and spleen from rabbits immunized intracutaneously was studied as follows suspensions with 1?0 mill/ml were prepared in the same way as used for cultivation but the cells were immediately disrupted by freezing and thawing three times After centrifugation the media were concentrated and analysed with immunodiffusion and Coomb s test

For studies on *de novo* antibody synthesis *in vitro* 0 5 mC of dl valine 1 C[14] (The Radiochemical Centre Amersham England) with a specific activity of 33 9 mC/mM was added to the depot medium in the flask and to the culture medium in the tubings This gave a ratio between radioactive and non radioactive valine of appr 1 15 The radioactivity of the media was 1 85 μC/ml The concentrated culture media were studied with immunodiffusion in the usual way except that 1 3 *per cent* agar was used (cf *van Furth* 1964) The agar slides were washed and dried in the standard way Stripping film autoradiographs using Kodak AR 10 plates was then performed with an exposure time of three weeks

RESULTS

Studies on Cell Survival during Cultivation

Cell suspensions from three rabbits were cultured with the use of a cell concentration of 60 mill/ml but orcein staining showed that only 43 54 mill/ml looked healthy at the start of the cultivation After four days in culture 13–24 mill/ml looked healthy after eight days 5–6 mill/ml after 12 days 1–3 mill/ml Autoradiography studies showed strong incorporation of valine H[3] in cells that looked healthy when stained with hematoxylin A pronounced cell degeneration thus occurs during cultivation only relatively few cells surviving

Studies on Antibody Content in Cells and Media after Cultivation

1) Adjuvant treated controls Cell suspensions from three rabbits were divided into 13 parts which were cultured for four or ten days in cell concentrations that varied from 20 to 100 mill/ml of culture medium The concentrated culture media gave no precipitates with human gamma globulin in immunodiffusion studies and Coomb s test was negative with two concentrated culture media studied The lymphoid cell suspensions especially after ten days cultivation also contained large cells that in phase contrast microscopy had the appearance of macrophages Some of them showed a dark green fluorescence at immunofluorescence microscopy but this colour differed from the specific apple green fluorescence This phenomenon was seen also in the two types of control slides (see Material and Methods)

2) Intravenously and intraperitoneally immunized rabbits and intracutaneously immunized rabbits heterogenous group These were used in a pilot study for investigating the possibilities of demonstrating antibody in the cells and culture media under various experimental conditions In all 14 different cell suspensions were cultured from the nine rabbits Fig 5 shows the results of immunodiffusion studies

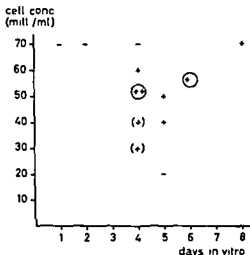


Fig 5

Results of immunodiffusion studies on concentrated culture media Rabbits belonging to the intravenously and intraperitoneally immunized group and the intracutaneously immunized heterogenous group
+ denotes distinct precipitates permitting reaction of identity (+) denotes faint precipitates not permitting reaction of identity — denotes no precipitates
Two encircled results indicate that two cell preparations (lymph node and spleen) from the same rabbit have been studied separately

Clear cut precipitates were found in seven experiments all gave a reaction of identity with the corresponding serum antibody In a further two experiments weak precipitates were obtained too indistinct to permit a reaction of identity In three of the seven concentrated media that gave a strong precipitate in immunodiffusion immunoelectrophoresis showed γG precipitates corresponding to those found in the immune sera of the same rabbits Coomb s test was performed on three concentrated culture media that gave precipitates in immunodiffusion two clear cut and one weak and indistinct The former showed titers of 1 1280 and 1 2560 the latter a titer of 1 640 Immunofluorescence studies were made on cells from four cultures with positive immunodiffusion results and from the four negative cultures Parts from these cell suspensions were also studied with immunofluorescence before the cultivation was begun Cells with an apple green fluorescence judged specific were found in slides from all parts studied except one taken before the start of the cultivation In this only very few cells with an apple green fluorescence were seen this part was therefore scored dubious After two or more days cultivation of this suspension the investigation gave a clear cut positive result All control slides (see Material and Methods) were negative Macrophages with a non specific dark green fluorescence as described above were found in all types of slides

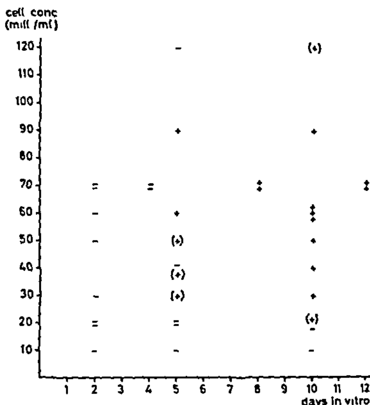


Fig 6

Results of immunodiffusion studies of concentrated culture media Rabbits belonging to the intracutaneously immunized homogenous group For symbols see Fig 5

3) Intracutaneously immunized rabbits homogenous group This group was used to study the influence of cell concentration and culture duration on the presence in the culture media of antibody demonstrable by immunodiffusion For this reason other variables were standardized source of cells immunization schedule and antigen dose With cell preparations from these eight rabbits 31 experiments were performed Fig 6 reports the results Five days was the shortest culture period that resulted in clear cut precipitates—cell concentrations were 60 and 90 mill/ml values below or above giving negative or indistinct results With increasing duration of cultivation positive results were obtained also with lower cell concentrations With all eight cell preparations positive results were obtained in at least one experiment All cell suspensions studied were thus able to produce antibody *in vitro* The negative results obtained can therefore be explained by too short culture periods or unsuitable cell concentrations Reaction of identity between antibody in immune serum and in concentrated culture medium could be demonstrated with materials from all eight rabbits Immunoelectrophoresis was performed on positive media con

centrates from cultures from all these cell preparations and in two γG precipitates were identified corresponding to those found in the sera from the same rabbits Seven concentrated culture media from two cell preparations were studied with Coomb s test Medium from one cell preparation was negative after cultivation for two days when studied with both immunodiffusion and Coomb s test After cultivation for five and ten days however the concentrated media showed pre cipitates in immunodiffusion and Coomb s test showed a titer of 1 20 Media from the second cell preparation were negative after two and four days cultivation but after eight and 12 days *in vitro*, precipitates were obtained and Coomb s test showed a titer of 1 80 Immunofluor escence studies were made on cell preparations from four rabbits be fore and after cultivation In three positive results were found both before and after cultivation In one cells taken before cultivation were dubiously positive but after two four and eight days cultivation they were unquestionably positive After 12 days cultivation the result was again scored dubious because fluorescent cells were few

Studies on de novo Synthesis of Antibody in vitro

Antibody observed in the culture media may be released from cells that have produced antibody already *in vivo* The antibody content of washed cell suspensions prepared from the regional lymph nodes and the spleen was therefore studied Four rabbits that had been injected intracutaneously with human gamma globulin three times during 1–2 months were used The titers of the sera at Coomb s test exceeded 1 4000 The cells from two cell preparations were disrupted in the serum Parker medium After centrifugation to remove cell debris and after the usual concentration of the medium antibodies were detected by immunodiffusion and these gave a reaction of identity with the corresponding serum antibodies The concentrated media gave titers of 1 320 and 1 2560 at Coomb s test Cell preparations from the other two rabbits gave after disruption no precipitates but Coomb titers of 1 320 Immunofluorescence was positive for all four cell preparations

Another three rabbits immunized as the homogenous group gave serum titers at Coomb s test of 1 16 1 128 and 1 512 Cells disrupted in the serum Parker medium gave after concentration no precipitates and Coomb s test was negative at 1 20 Immunofluorescence was posi tive for all three cell preparations

Thus washed cell preparations from rabbits with high serum anti body titers contained already *in vivo* considerable amounts of antibody and a release of such antibodies during cultivation is perhaps not negligible Cell preparations from rabbits immunized according to the schedule used for the homogenous group gave no release detectable by the antibody assay methods used Such cell preparations were there



Fig 7

Autoradiogram of immunodiffusion in agar performed on medium from a culture to which valine C^{14} was added Concentrated culture medium in the central well and the antigen dilutions in the peripheral wells Strong specific labelling at the site of precipitates Weak non specific labelling around central well

fore studied for further verification that antibody obtained in the culture media was synthesized *in vitro* A radioactive trace method was used for this purpose

Cell suspensions from one adjuvant treated control rabbit and from two rabbits belonging to the intracutaneously immunized homogenous group having Coomb titers of 1 128 and 1 512 were all cultured in media containing valine C^{14} A culture period of ten days and cell concentrations of 60 mill/ml were used Both concentrated culture media from the cell preparations taken from the rabbits immunized with human gamma globulin showed precipitates in immunodiffusion Autoradiography proved that both precipitates contained radioactive material (see Fig 7) The cell suspension from the adjuvant treated control rabbit was divided into two parts one was cultured with autologous serum in the standard way the other in a mixture of the immune sera from the two rabbits immunized with human gamma globulin Precipitating antibodies could as expected be demonstrated only in the medium made up with immune sera Autoradiography was negative for both parts Thus no incorporation *in vitro* of radioactive material took place into the antibody synthesized *in vivo* and no trapping of radioactive material occurred at the formation of the precipitates in the control experiment

DISCUSSION

This paper describes a simple method for cultivation of lymphoid cell suspensions for many days When cells were taken from rabbits immunized with human gamma globulin antibody was produced in an amount that permitted analysis with immunodiffusion and in some cases with immunoelectrophoresis Most workers in this field think that a considerably better production of antibody is obtained in organ cultures than in cell suspension cultures *Stavitsky* (1958) *Askonas & Humphrey* (1958) and others stress the unavoidable trauma that cells are exposed to when a cell suspension is prepared *Helmreich et al* (1961) quantitized the probable cell damage caused at the preparation of lymphoid cell suspensions and found this to represent a release of not more than five per cent of total cellular aldolase activity In the present study antibody production has been obtained from cultures prepared by simple teasing of the lymph nodes and spleens *Pospisil* (1963) suggested that the compact character of lymphatic tissue transferred to tissue cultures permits differentiation processes or promotes cell modulation processes in some other way *Halliday & Garvey* (1964) and *Bussard & Hannoun* (1966) *inter al* also mention this possibility *Michaelides & Coons* (1963) point out that the difference can be explained if aeration is important in antibody synthesis *in vitro* Recently the importance of aerobic conditions has been stressed by *Svehag* (1965) and *Globerson & Auerbach* (1966)

In order to obtain better results with culture of cell suspensions methods have been worked out to increase exchange between cells and gas and fluid phases of the culture *Steiner & Anker* (1956) placed cell suspensions from immunized animals on a semi permeable membrane in a thin fluid layer to obtain a good exchange with the gas phase above Below the membrane was put a depot medium that was constantly stirred with a magnetic stirrer Antibody production was obtained for a few days The antibody produced *in vitro* was therefore accumulated in a small fluid volume and could be directly demonstrated with immunoprecipitation This culture method however was judged difficult to handle by for instance *Stevens & McKenna* (1958) The method has recently been used by *Gurvich et al* (1965) and in a modified form by *Nelin* (1965)

Amis (1962) introduced a still more complicated culture method This combined Trowell s principle of maintaining the tissues near the surface of the medium as well as certain aspects of Steiner and Anker s chamber Antibody formation could be demonstrated with microquantitative precipitin test even after as long as 13 days cultivation of lymphoid cell suspensions from immunized animals

Svehag (1965) cultured lymphoid c ll suspensions from immunized animals on membrane filters glued t he top of lucite cylinders each placed in a depression of a plastic tra Culture medium was placed in

the depressions so that the cell suspensions were situated in the gas fluid interphase. With the sensitive technique of virus neutralization antibodies could be demonstrated in the media after 2–3 weeks cultivation.

In the culture method presented in this paper cell suspensions are not in close contact with the gas phase. Nevertheless, good antibody production has been obtained for many days; therefore the aeration might not be such a critical feature as previously suggested.

As stressed in the introduction, it is necessary to differentiate antibody synthesized *de novo in vitro*, and antibody synthesized *in vivo* and merely released *in vitro*. In early studies the antibody content of media where living cells had been cultivated was compared with that of media where dead cells had been included or with that of extracts of non cultivated cells (cf. *Fagraeus* 1948). When such a method was used in present context, it was found that cell suspensions from rabbits with high serum antibody titers contained considerable amounts of antibody; this might be passively released owing to the pronounced cell degeneration during cultivation. Cell preparations from rabbits with low antibody titers in serum (homogenous group) did not contain enough antibody for demonstration by the used methods. More recently the *inhibiting effect of chloramphenicol, actinomycin D or puromycin* has been used for indirectly proving the *de novo* synthesis of antibody *in vitro*. Recently *Stavitsky & Gusdon* (1966) pointed out that actinomycin D can stimulate or inhibit antibody synthesis *in vitro* depending on the concentration used. *Stavitsky* (1963) has stressed that *measurement of the extent of the incorporation of radioactive amino acids into antibody is the only reliable distinction between de novo synthesis and release of antibody. Incorporation can be directly demonstrated after precipitation with the homologous antigen. Most authors have then used co precipitation with non radioactive antibody*, a sensitive method that might contain large sources of error due to absorption of non specific radioactivity (cf. *Stavitsky* 1961). *Steiner & Anker* (1956) obtained amounts of antibody large enough to permit direct precipitation with the antigen and demonstration of radioactivity of the produced antibody. The same goal was reached with the method described in the present paper and indirect methods of demonstration of antibody synthesis could be avoided.

For studying the relative amounts of cells taking part in antibody production *immunofluorescence methods can be used. Using this method Patterson et al.* (1964) found a very high proportion of antibody containing cells—sometimes nearly all the cells in their slides were fluorescent. In the present study relatively few scattered cells contained antibody as demonstrated by this method. No obvious explanation can be given for these divergent results, but it has to be pointed out that different experimental systems were used. (See also *Nilsson* 1967). Usually no significant changes in proportions of such cells oc

curred during cultivation In only two experiments did a dubious positive result before cultivation become definitely positive after a few days cultivation of the cells The immunofluorescence results were apparently uninfluenced by cell concentration in the cultures This contrasts with the above mentioned experiences with antibody analysis of the culture media Non specific fluorescence in macrophage like cells could slightly complicate the evaluation but it was never a serious problem

The advantages of the present method for cultivation of lymphoid cells can be summed up in the following points

1 the culture method is simple and can easily be modified for various experimental purposes

2 it permits the cultivation for many days of cell suspensions with high cell concentrations

3 when cells are taken from rabbits immunized *in vivo* antibody production occurs in amounts large enough to permit detection with immunoprecipitation in agar gel and sometimes with immunoelectrophoresis Direct evidence of synthesis *in vitro* can therefore be obtained from incorporation experiments with radioactive amino acids

4 the method permits removal of portions of both culture medium and cells at arbitrary intervals during cultivation

SUMMARY

A simple method for cultivation of rabbit lymphoid cell suspensions for many days is described Cells taken from rabbits immunized *in vivo* with human gamma globulin produced antibody *in vitro* in amounts large enough to permit analysis with immunoprecipitation in agar *De novo* synthesis *in vitro* could be directly proved with incorporation of radioactive amino acid into the immunoglobulin in the culture medium

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THE MONONUCLEAR CELL INFILTRATE IN ALLERGIC CONTACT DERMATITIS

3 *Selective Accumulation of Cells from Lymph Nodes*

By

Sture Lidén

Received 11 ii 67

A selective accumulation in allergic contact reactions of lymphoid cells labelled early during the sensitization process has been demonstrated (4 9) These selectively accumulating cells do not constitute any major part of the infiltrating lymphoid cells It is possible however that they represent a hypothetical sensitized cell clone induced to proliferate by the sensitization The origin of these labelled cells has not been fully clarified By transfusion of thymic cells *Kosunen* (6 7) found but insignificant numbers lodging in reactions of tuberculin type in rats In an earlier experiment in this series with local thymic labelling the same results were reached in allergic contact dermatitis (10) Locally labelled bone marrow cells on the other hand were found to accumulate selectively in these reactions (11)

Of the peripheral lymphoid organs the lymph nodes have been ascribed a vital role for the elicitation of contact hypersensitivity (2) In the lymph nodes regional to sensitization with contact allergens an increase in the number of large pyroninophilic cells has been found (12 16) The maximal numbers were found on the 4th to 5th days after the sensitizing application Labelling data indicate that these cells develop into small lymphocytes within the first days (14) It seems reasonable that the lymphocytes thus derived are carriers of immunologic information important for the elicitation of the allergic contact dermatitis In the above mentioned works *Kosunen* also transfused cells from lymph nodes regional and non regional to sensitization with tuberculin between inbred rats After *in vitro* labelling with tritiated leucine he found a selective but non specific accumulation of cells from the regional lymph node compared with the non regional

The work was supported by the Swedish Medical Research Council

node (6) After *in vivo* labelling with tritiated thymidine on days 10–12 after sensitization cells collected and transfused on day 14 showed in addition a certain degree of immunological specificity (7)

In a series of experiments on the mononuclear cell infiltrate attempts have been made to achieve what could be called an ideal labelling of different parts of the lymphoid system Ideal means here that the actual cells are labelled in such a way that they can be traced within the labelled animal itself In this way the handling of the cells during preparation of cell suspensions and the effects of the transfusion on cells and recipients are avoided In the present experiment use was made of a method for local labelling of lymph nodes in an attempt to trace cells labelled during the period of maximal large pyroninophilic cell proliferation in the lymph nodes regional to contact sensitization

The purpose of the work was to compare cells from lymph nodes regional and non regional to contact sensitization with 2 4 dinitro chlorobenzene (DNCB) with respect to their tendency to accumulate selectively in contact allergic reactions The immunologic specificity of this accumulation was also studied by comparing the percentage of labelled lymphoid cells *in reactions to DNCB (used for sensitization of* locally labelled lymph nodes) with reactions to 2 phenyl-4 ethoxy methylene 5 oxazolone (oxazolone) used for sensitization in a non labelled lymph node station

MATERIAL AND METHODS

Animals

Twelve white male guinea pigs about 6 weeks old and obtained from the same breeder were used At the outset the 6 animals labelled in the sensitization area weighed 211 ± 7 g (S D) and the 6 animals labelled contralaterally 08 ± 13 g

Sensitization

The animals were double sensitized with 2 4-dinitrochlorobenzene (DNCB)[1] and 2 phenyl 4 ethoxymethylene-5-oxazolone (oxazolone) This latter substance was synthesized in the following way according to a method given by *Barber & Slack* (1) Hippuric acid and ethyl orthoformate were heated with acetic anhydride for one hour under reflux The low boiling material was removed by a vacuum pump On cooling the residue solidified After four recrystallizations from light petrolatum in the cold (+4 C and −20 C) almost colourless (slightly pinkish) crystals were obtained Determination of the melting point gave a value of 98 0 100 5 C (compared with 97 –98 C according to *Barber et al*)

1 per cent DNCB in acetone (w/v) was applied to the skin of the left axillary region outlined as described earlier (8) 0 2 ml of this solution was distributed over the entire area on day 0 and 1 and 0 3 ml on day 2 This mode of sensitization resulted in but slight necrosis of the treated skin

The oxazolone was applied as a 10 per cent solution in absolute alcohol of which 0 2 ml was applied to both sides and to the hairless base of one ear on day 0 only This substance is completely atoxic even at this concentration However the *appearance of the sensitization* was observed between days 6 and 8 as an erythema and oedema of the sensitization ear

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1 Merck AG Darmstadt Germany

Labelling

The labelling was performed with fresh methyl tritiated thymidine[1] (H^3–TdR) with a specific activity of 1.9 c/mM. The marker was injected intradermally in order to induce local lymph node labelling (8, 13, 15). In a preliminary experiment 2 groups of guinea pigs with 3 animals in each group were given intradermal injections in one of the axillary areas. Each animal was injected twice daily until 7 injections had been given. The animals in the two groups received respectively 1 µc and 2.5 µc of H^3 TdR diluted in 100 µl of 0.9 per cent saline at each labelling occasion. The animals were killed 1 hour after the last injection. The labelling intensity in the lymph nodes regional to labelling after both these doses was fairly high but not optimal for the present experiment even if the maximal individual grain counts exceeded 50. In the control lymph nodes the maximal grain count was 8 in the group which received 2.5 µc per injection. A few heavily labelled cells present in the control lymph nodes were regarded as migrants from the locally labelled lymph nodes.

Considering the interval of 6 days between the last labelling injection and biopsy in the main experiment the individual labelling doses were increased to 5 µc of H^3 TdR diluted in 100 µl of 0.9 per cent saline. The H^3 TdR solutions were never more than 17 hours old when the injections took place. The 5 µc were injected on 8 different occasions three times a day (injection times 8 a.m., 3 p.m. and 11 p.m.) beginning at 3 p.m. on day 2 and ending at 11 p.m. on day 4. Six animals were labelled in this way in the left axillary area, *i.e.* the area of sensitization, and six other animals in the right axillary area. All animals were given a short and superficial ether narcosis (duration about 2 minutes at the most) on every labelling occasion. No side effects were noted from the treatment.

Testing

0.200 per cent DNCB and 0.1% per cent oxazolone dissolved in absolute alcohol were used as test substances. On day 20 µl of the test solutions were applied to a circular area measuring 2 cm on the electrically shaved hind parts of the mid flanks. The oxazolone test and one of the DNCB tests was applied 24 hours and the other DNCB test 12 hours before taking the biopsies on day 11.

Skin Biopsies, Histologic and Autoradiographic Technique

Skin biopsies were taken from the three different test sites and from untreated control skin in the vicinity of the 24 hour DNCB test. All biopsies were taken at the same dorsoventral level. They were fixed in formalin and embedded in paraffin. Sections cut at 5 µ from all four biopsies of each animal were placed on the same slide together with a section from a non labelled animal. The positions of the different skin types were changed in order to compensate for possible systematic differences in autoradiographic quality in various parts of the film. For autoradiography Kodak AR 10 stripping film was used. The exposure time was 8 weeks. The slides were stained using haemalum according to Mayer after development of the film.

A more detailed account of the procedure hitherto described has been given in a previous paper (9).

Evaluation of Autoradiogram

The percentage of labelled lympho[illegible] cells in corium and epidermis and the number of grains over the nu[illegible] f the[illegible] cell was determined. The lymphoid cell were defined according to *Groth* (3[illegible] Macrophages as defined by *Kosunen, Waksman, Flax & T[illegible]* (5) were [illegible] the lymphoid cells in corium. The limit for cla[illegible] a cell a[illegible] the same as that employed in the previous investig[illegible] n this [illegible] d cells in corium showing 3 grains or more and lym[illegible] grains or more were regarded a[illegible] labelled. Cont[illegible] the backg[illegible] as accompl[illegible]hed by counting of 100 lymphoid cell[illegible] um of the [illegible] ctions on 3[illegible] different slide. Of the c

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[1] Schwarz [illegible] rch In[illegible] USA

3,200 cells 0.5 per cent had 3 grains or more. This is well below the 2 per cent chosen as upper limit. No differentiation between the different lymphoid cell types was made. In this way 100 lymphoid cells in the epidermis and 1 000 in the corium were counted in sections from each of the four different biopsies taken from every animal. As a rule parts of 8 sections were counted from each biopsy. The maximal grain count number over the skin epithelial cells was determined by screening at least one section from every animal. In an additional counting a further 100 and 1 000 lymphoid cells were counted in the epidermis and in the corium respectively from each of the different biopsies. This time only cells with a grain count exceeding the maximal grain count of the epithelial cells in epidermis were recorded. These cells were considered as probable migrants from the locally labelled lymph node stations.

TABLE 1

The Difference between the Percentage of Labelled Lymphoid Cells in Test Skin (T) and Control Skin (C) in Two Groups of Guinea Pigs Labelled Locally in Different Ways

Treatment	Animal	Per cent labelled lymphoid cells T–C: Corium DNCB 12 hrs	Corium DNCB 24 hrs	Corium Oxa 24 hrs	Corium Control	Epidermis DNCB 12 hrs	Epidermis DNCB 24 hrs	Epidermis Oxa 24 hrs	Epidermis Control
Labelling	1	2.0	1.1	0.7	4.7	−4	5	4	20
of lymph	2	3.3	4.0	3.9	3.6	13	4	8	13
nodes	3	1.7	[illegible]	2.9	2.2	3	12	8	10
regional to	4	−0.3	−0.3	−0.8	4.5	15	0	13	13
sensitization	5	0.6	0.4	0.8	3.9	5	−1	8	12
	6	0.2	0.5	1.1	2.9	0	2	[illegible]	13
Mean values		1.3	1.3	1.4	3.6	5.3	3.7	[illegible]	13.5
Labelling	1	0.6	2.0	0.4	[illegible]	4	7	0	14
of lymph	2	[illegible]	0.5	−0.5	3.6	8	8		13
nodes contra	3	5.9	1.9	3.3	2.6	4	4	[illegible]	17
lateral to	4	1.4	3.3	1.7	2.3	1	1	1	16
sensitization	5	−0.9	1.0	0.2	3.7	−4	1	8	21
	6	0.1	2.5	0.1	3.6	12	[illegible]	[illegible]	17
Mean values		1.5	1.9	0.9	3.1	3.2	3.9	[illegible]	[illegible]

In corium 1 000 lymphoid cells and in epidermis 100 lymphoid cells have been counted in each of the four biopsies from every animal.
DNCB = 2,4 dinitrochlorobenzene
Oxa = 2 phenyl 4 ethoxymethylene-5 oxazolone
The values of the control skin are presented in order to indicate the level of labelling.

RESULTS

The results from the countings recording lymphoid cells with 3 grains and more over their nuclei are presented in Table 1. The values of the test skins have been corrected by subtraction of the corresponding values of the control skin in order to compensate for possible differences in degree of labelling between the animals. Comparison between the animals labelled in the sensitization area and those labelled contra-

laterally shows no statistically significant differences for any of the skin types. Nor are there any significant differences between the percentages of labelled cells in the biopsies from the DNCB test sites and from the oxazolone test site. These countings do not even indicate a trend in any direction. The percentages of labelled cells in the test skins, however, show higher values than in control skin in most instances, even if statistical significance is not reached for all the different cell populations counted.

The lymphoid cell responses varied widely between the different reactions but were as a rule high. No correlation was found between the intensity of the reactions measured by this method and the increase in percentage of labelled cells in any of the groups.

The screening of epithelial cells in the epidermis for determination of their maximal grain count values showed a variation between 10 and 15 grains for the different animals. Cells with more than 10 grains were, however, rare.

TABLE 2

Numbers of Lymphoid Cells with ≧ 16 Grains

		DNCB 12 hrs	DNCB 24 hrs	Oxa 24 hrs	Control
Labelling of lymph nodes regional to sensitization	Corium	27 (2.3)	19 (1.6)	6 (0.5)	2 (0.2)
	Epidermis	20 (16.7)	4 (3.3)	8 (6.7)	0
Labelling of lymph nodes contralateral to sensitization	Corium	24 (2.0)	21 (1.8)	13 (1.1)	4 (0.3)
	Epidermis	7 (5.8)	2 (1.7)	5 (4.2)	0

From each of the two labelling groups 12 000 lymphoid cells have been counted in corium and 1,200 in epidermis from each of the four biopsy types.
Figures in brackets indicate per mille of labelled cells.

The results from the countings of cells showing a grain count exceeding that of the epithelial cells, i.e. 16 grains or more, are presented in Table 2. From each of the four biopsies 2 000 lymphoid cells in corium and 200 in epidermis were counted. In every type of test reaction and in both animal groups the test skin biopsies show an accumulation of these heavily labelled cells compared with control skin. The proportion of these labelled cells is larger in the corium of the DNCB tests than in the oxazolone tests. This applies to both labelling groups, however, even if the difference is less pronounced in the group labelled contralaterally to sensitization. The proportion of heavily labelled cells is generally higher in epidermis than in corium. The most obvious difference between the proportion of these cells exists in epidermis of the 12 hour and the 24 hour DNCB tests.

General microscopy of the sections yielded additional data. The majority of the labelled cells were typical lymphocytes (small, medium and large). Labelled cells which undoubtedly were macrophages were

observed but with a very low frequency. *Macrophages* with a grain count exceeding 15 were occasionally encountered.

DISCUSSION

In a previous investigation the radioactivity in the locally labelled lymph nodes in non-sensitized animals was found to decrease successively during the first week, at the end of which the level of the control nodes was approximated (8). Thus most of the cells labelled on days 2–4 probably have left the locally labelled lymph nodes at the application of the tests on day 10. As shown by *Macher* (12) the lymph nodes regional to contact sensitization with DNCB increase in weight during the sensitization. This increase begins at about the 4th day. The H^3-TdR injections given on days 2–4 thus may give labelling of more cells in the animals injected in their sensitization area than in the control group injected contralaterally to this. This fact emphasizes the importance of using the relationship between the autoradiographic values within the same labelling group when comparing the two groups with each other.

The number of lymphoid cells infiltrating the contact allergic reactions varies with the intensity of the reactions. Furthermore the cellular density of the infiltrate varies in different parts of the same biopsy, the lymphoid cell number being highest around vessels, as is the case in delayed hypersensitivity reactions in general (18). Because of these circumstances, systematical counting of a certain number of cells probably gives a more representative picture of the percentage of labelled cells than does counting of the number of labelled cells per view field (6, 7).

The increase above the control skin level in the percentage of labelled lymphoid cells in all tests but one shown in Table 1 is in agreement with results obtained in experiments using general labelling early during the sensitization process (4, 9). It is possible that also in the present experiment this is an effect of the general labelling resulting from the intradermal injections. If this is the case, it may disguise an immunologically specific accumulation of lymphoid cells.

The tracing of heavily labelled lymphoid cells, i.e. cells with a grain count which is larger than the maximal grain count in the epithelial cells of the epidermis, gives a somewhat different picture. These countings show that these cells are present in the test skin more frequently than in control skin, but also that they have a preference for epidermis compared with corium. With regard to the immunologic specificity no firm conclusions can be drawn, even if a tendency to larger differences between the DNCB tests and the oxazolone tests can be observed in the regionally labelled group than in the contralaterally labelled group. The most conspicuous finding is the pronounced difference between the numbers of heavily labelled cells in epidermis of

the 12 hour and the 24 hour DNCB tests in the regionally labelled group. This result tallies well with the concepts of cytotoxicity reviewed by *Waksman* (17) i.e. that sensitized cells are destroyed on contact with the specific antigen. It might be questioned if all cells with 16 grains or more really are derived from the locally labelled lymph nodes. It is possible that some cells labelled by the systemic labelling effects of the local labelling attempts can reach this degree of labelling. On the other hand most of the heavily labelled cells have 20 to >50 grains, numbers which are most unlikely to result from general labelling.

In the present investigation a considerable proportion of the labelled lymphoid cells were small – medium lymphocytes. Only occasionally heavily labelled macrophages were noted. The pattern of labelling is thus different from that observed in a previous experiment with labelling about 3 days before taking the biopsies 48 hours after testing, where the labelled population was dominated by large lymphocytes and macrophages (11). If this difference is caused by the varying labelling techniques, by the times chosen for labelling or by the different ages of the test reactions is not possible to determine on the basis of available data.

The conclusions of this experiment are based mainly on the results obtained from the countings of the heavily labelled lymphoid cells which are presumed to be largely migrants from the locally labelled lymph nodes. These cells make up part of the mononuclear cell infiltrate in allergic contact dermatitis. They are found in a larger percentage in epidermis than in corium. They constitute a higher percentage of the lymphoid cells in test skin than in control skin, i.e. they selectively accumulate in the test skin. No clearcut evidence for an immunologic specificity of this selective accumulation has been found. The findings suggest however that there may be an early accumulation of immunologically specific cells at least in the epidermis and that these cells disappear later, possibly as a result of a cytotoxic action of the antigen on those cells. The general labelling pattern showed that lymphoid cells of all sizes were labelled.

SUMMARY

Albino guinea pigs were doubly sensitized with two different contact antigens DNCB and oxazolone. At the time of the most pronounced proliferation of large pyroninophilic cells in the lymph nodes regional to the sensitization the animals were labelled with tritiated thymidine. In one group of animals a practically local labelling of the lymph nodes regional to sensitization with DNCB was performed. In another group such local labelling was given to the lymph nodes contralateral to those regional to sensitization. No local labelling was given to the lymph nodes regional to sensitization with oxazolone. Tests were ap

plied with both antigens and biopsies were taken after 12–24 hours. Autoradiographical analyses showed that lymph node cells participate in the mononuclear cell infiltrate of the allergic contact reactions. The lymph node cells were more frequent in test skin than in control skin, indicating a selective accumulation at the test sites. An immunological specificity of this accumulation could not be established. The lymph node cells showed a preference for epidermis compared with corium. The lymphoid cell infiltrate contained more labelled cells of small-medium size than the infiltrate of tests in a previous experiment where the animals were labelled shortly before testing.

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EXPORT AND IMPORT OF LYMPHOCYTES IN THE THYMUS DURING STEROID INDUCED INVOLUTION AND REGENERATION

By

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Certain steroids particularly the glucocorticosteroids cause characteristic involution of the thymo lymphatic tissue (*Selye* 1936 *Ingle* 1940 *Dougherty & White* 1945) This involution being part of the stress syndrome (*Selye* 1951) is most pronounced in the thymus The mechanism responsible for this thymic atrophy has been studied by many authors with divergent results in certain respects Histologically nuclear pyknosis and lymphocytolysis are reported in the thymic cortex *in vivo* as well as *in vitro* during the first hours after administration of corticosteroids (*Dougherty & White* 1943 1944 1945 *Schrek* 1949 *Feldman* 1950 *Baker et al* 1951 *Ehrich & Seifter* 1953) The corticosteroids are also stated to inhibit the synthesis of protein and nucleoproteins especially DNA and to decrease the mitotic activity of the thymo lymphatic tissue (*Dougherty & White* 1945 *Studer* 1950 *Engel* 1951 *Hull & White* 1952 *Pfeiffer et al* 1952 *Kass et al* 1953 *Kit & Barron* 1953 *Robbins* 1955 *Clark & Stoerk* 1956 *Blecher & White* 1958 *Lundin* 1958 *Ishidate & Metcalf* 1963 *Bellamy et al* 1966 *Knutson & Lundin* 1966)

In the blood a deficiency of lymphocytes is reported in steroid treated animals (refs *Dougherty* 1959) This may be due to intravasal lysis of lymphocytes and/or to reduced production and delivery of lymphocytes from the thymo lymphatic system In the guinea pig the thymus is a major contributor to the circulating small lymphocytes (*Ernstrom & Larsson* 1966a) and in this animal the export of lymphocytes from the thymus under different conditions can be evaluated by a direct comparison between the lymphocyte content of the arterial blood and that of the thymic venous blood In the present study the migration of lymphocytes to and from the thymus was investigated during steroid induced involution and regeneration of the thymo-lym

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phatic tissue Furthermore the experiments were extended to include the effect of a corticosteroid on the circulating lymphocyte populations of thymectomized guinea pigs

MATERIAL AND METHODS

Totally 314 male guinea pigs with an initial weight of 237 ± 9 g (mean ± S E) were used The animals were fed on cabbage turnips carrots and vitamin pellets They were divided into four main groups (number of animals in brackets)

Non operated controls injected with saline (43)
Non operated animals injected with prednisolone (120)
Thymectomized controls injected with saline (75)
Thymectomized animals injected with prednisolone (76)

Thymectomy was performed under local anaesthesia (subcutaneous infiltration of 0 5 per cent Xylocaine® Astra) according to the technique of *Gyllensten* (1953)

Prednisolone (Ultracortenol® Ciba) was administered i p in a dose of 50 mg/kg b w In the thymectomized guinea pigs the injection was given immediately after operation Examinations were made 1 3 and 6 hours and 1 3 6 and 9 days after treatment about 15 animals at each time The investigations were made between 9 a m and 4 p m The animals were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg/kg b w i p)

In the non operated animals the thymus was exposed and the largest of the thymic veins ligated and incised A sample of blood was taken in a heparinized pipette (Heparin® Vitrum 5000 IU/ml) for supravital staining of white blood cells for white cell counts and for preparation of blood smears

The right carotid artery was incised near the origin of the thymic artery and samples of arterial blood were taken for analysis as above Immediately afterwards the artery was ligated The confluence of the thoracic duct and the left subclavian and jugular veins was then exposed essentially according to the technique of *Reinhardt & Yoffey* (1957) The thoracic duct was punctured and a sample of lymph was collected in a pipette for supravital staining The whole procedure was performed under a dissection microscope

The supravital specimens of blood and lymph were stained with Janus green B and neutral red (see *Ernström & Larsson* 1966 b) and were immediately examined in a light microscope at 1 000 × magnification Differential counts were made of lymphocytes with different mitochondrial content 100 cells per specimen being counted The lymphocytes were registered in six classes cells with 0–5 6 10 11 15 16–20 21–30 and > 30 mitochondria For convenience 0 10 11 20 and > 20 mitochondria per cell are denoted as low medium and high mitochondrial content (*MC*) *respectively*

For determination of the absolute number of white blood cells 25 mm^3 of blood was diluted with 475 mm^3 of Toisson s solution (containing methyl violet for staining of white cells) The number of cells was counted in a Bürker counting chamber 288 squares (corresponding to 0 00625 mm^3 each) being counted for each sample

The blood smears were fixed in methanol for 5 minutes and stained with Giemsa for 12 minutes They were examined in a light microscope and differential counts were made of lymphocytes monocytes and granulocytes

The results were analyzed statistically by Student s *t* test The comparison between the percentage and number of lymphocytes in thymic vein and carotid artery blood was performed by statistical analysis of all the differences in the individual animals The *p* values < 0 001 < 0 01 and < 0 05 are denoted as highly significant significant and almost significant respectively

RESULTS

No mortality occurred The increase in body weight was about 5 g a day irrespective of treatment or operation

Control Animals

In the controls the lymphocyte content per mm³ of blood from the thymic vein exceeded that from the carotid artery ($p<0.001$) again proving the existence of a net emigration of thymic lymphocytes in normal young guinea pigs (see *Ernstrom et al* 1965) A subdivision of the lymphocyte population based on the mitochondrial content of the cells resulted in the following veno arterious difference in number of cells per mm³ of blood passing through the thymus the statistical analysis being based on the differences in the individual animals (mean ± S E) Lymphocytes with low mitochondrial content 629±167 ($p<0.01$) Lymphocytes with medium mitochondrial content 122±67 Lymphocytes with high mitochondrial content —8±9

As the mitochondrial content is correlated to the size of the lympho cytes (*Wiseman* 1931 *Fichtelius & Larsson* 1961 *Ernstrom & Larsson* 1963) this once more proves that the emigrating thymic cells are small lymphocytes and that no net emigration of large lymphocytes occurs (see *Ernstrom & Larsson* 1966a)

A higher number of granulocytes per mm³ of blood was noted in the carotid artery than in the thymic vein ($p<0.001$) indicating an immigration of granulocytes into the thymus This was not found to be statistically significant in earlier experiments (*Ernstrom & Lars son* 1966a)

In the thoracic duct lymph the medium sized lymphocytes (with medium MC) dominated (65 1±0 8 per cent)

TABLE 1

Percentage and Number of White Cells in Carotid Artery Blood of Control and Steroid Treated Guinea Pig Mean ± S E

Interval after steroid injection	No of animals	Lymphocytes %	Lymphocytes No/mm³	Monocytes %	Monocytes No/mm³	Granulocytes %	Granulocytes No/mm³
Controls	43	64 0±1 2	1710±133	3 2±0 2	93±12	32 8±1 8	932±104
1 hour	19	59 3±3 3	1713±213	2 6±0 3	75±15	38.2±3 3	1121±164
3 hours	19	33 8±3 5	1710±23	2 3±0 4	110±16	64 0±3 5	3261±411
6 hours	19	37 6±4 5	1468±153	2 7±0 3	131±23	59 6±4 5	3152±594
1 day	18	62 0±3 2	1933±222	2 5±0 2	79±18	33 5±3 2	1140±172
3 days	15	66.2±2 4	1598±145	2 2±0 3	54± 8	31 6±2 4	791±103
6 days	15	63 5±4 0	1916±194	2 9±0 3	91±15	33 6±4 0	1047±157
9 days	15	69 9±2 1	2100±207	2 0±0 2	61±10	28 1±2 0	870±123

Prednisolone Treated Animals

No general lymphocytopenia occurred (Table 1) However subdivi sion of the lymphocyte population into cells with low medium and high mitochondrial content (MC)—corresponding to small medium sized and large lymphocytes—revealed a decreased number of small

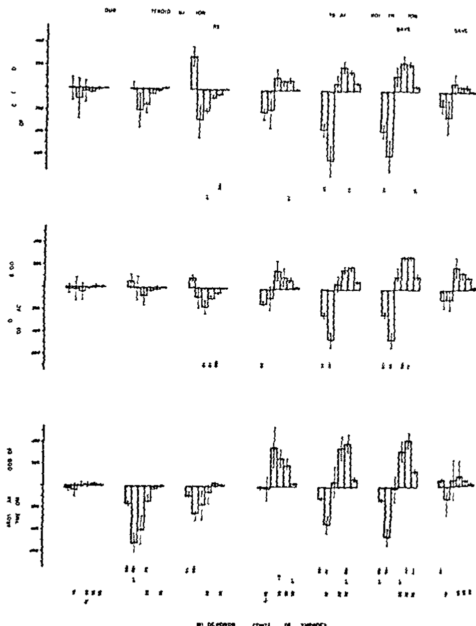


Fig 1

Steroid induced changes in numl er of lymphocytes with different mit chondrial content per mm^3 of blood in the thymic vein an l in the carotid artery f guinea pigs with an intact thymus and in the carotid artery of thymectomized guinea pigs The bars in licate the mean difference ($\pm$S E) between the numl er of l ymphocytes of different categories in the steroid treated non operated an l thym mized animals on the one hand and that in the normal contr l and the c ntr l treated thymectomized animals at the corresponding interval after operati n n the other hand

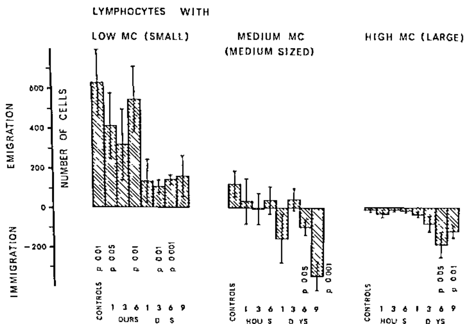


Fig 2

Thymic emigration and immigration of lymphocytes in guinea pigs normally and during steroid induced thymic involution and regeneration (1 3 6 hrs 1 3 6 and 9 days after injection of a thymolytic steroid) determined as the difference between the number of lymphocytes in the thymic vein and that in the carotid artery The lymphocytes are subdivided into cell with low medium and high mitochondrial content (MC) Mean ±S E

lymphocytes and an increased number of larger cells per mm³ of blood during the late phase (1 3 and 6 days) after steroid injection (Fig 1) The most pronounced change was seen among the smallest lymphocytes with 0–5 mitochondria per cell which were decreased to 15 per cent of the control value 3 days after steroid administration At 9 days no significant deficiency of small lymphocytes was found whereas an increased number of larger lymphocytes persisted (Fig 1)

The thymic veno arterious difference in number of lymphocytes with low MC (small cells) was decreased in the steroid injected animals in comparison to that in the controls (Fig 2) This decrease in number of small lymphocytes emigrating from the thymus was significant during the late phase (1 3 6 and 9 days) after prednisolone injection (Table 2) In contrast to normal guinea pigs the animals investigated 6 and 9 days after steroid injection had a larger number of lymphocytes with medium ($p<0.05$ and $p<0.001$) and high MC ($p<0.05$ and $p<0.01$) in blood from the carotid artery than in blood from the thymic vein (Fig 2) This indicated a net immigration of medium sized and large lymphocytes into the thymus during the regenerative phase after steroid induced thymic involution (for histological details of predni

solone induced involution and regeneration of the thymus in the guinea pig see *Gyllensten* 1962 *Ernstrom & Gyllensten* 1965)

At 3 and 6 hours after steroid injection pronounced granulocytosis appeared the number of granulocytes being 350 and 338 per cent of the control value (Table 1) In all the steroid treated animals an excess of granulocytes was found in carotid artery blood in comparison to thymic vein blood

TABLE 2

Thymic Emigration (Veno Arterious Difference in No of Cells/mm³ of Blood) of Small Lymphocytes Characterized by Low Mitochondrial Content in Controls and Steroid Treated Guinea Pigs A Decreased Output of Small Lymphocytes is Demonstrated 1 3 6 and 9 Days after Steroid Injection Mean Difference ± SE

		Interval after steroid injection						
	Controls	1 hour	3 hours	6 hours	1 day	3 days	6 days	9 days
No of emigrating small lymphocytes	629±167	413±168	390±181	548±164	135±109	111±36	139±23	156±106
t	3.77	2.46	1.77	3.34	1.24	3.08	6.04	1.47
df	15	9	11	11	9	10	10	10
p	<0.01	<0.05		<0.01		<0.05	<0.001	
Steroid induced decrease in no of emigrating small lymphocytes		216±237	309±246	81±234	494±199	518±171	490±169	473±198
t		0.91	1.26	0.35	2.48	3.03	2.90	2.39
df		24	26	26	24	25	25	25
p					<0.05	<0.01	<0.01	<0.05

At 6 hours after steroid injection the percentage of small thoracic duct lymphocytes (with low MC) was increased ($p<0.001$) and that of medium sized and large lymphocytes (with medium and high MC) correspondingly decreased ($p<0.001$ and $p<0.001$) At 3 and 6 days the percentage of small lymphocytes in the lymph was decreased ($p<0.001$ and $p<0.001$) and that of large lymphocytes markedly increased ($p<0.001$ and $p<0.001$) (Fig 3)

Thymectomized Animals

The total number of blood lymphocytes was decreased 1 hour after operation ($p<0.001$) the decrease comprising all subgroups of lympho-

Fig 3

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph of intact guinea pigs treated with a thymolytic steroid and of thymectomized guinea pigs treated with saline or steroid at different intervals after steroid injection and thymectomy Mean ±SE The arrow indicates injection of steroid and thymectomy

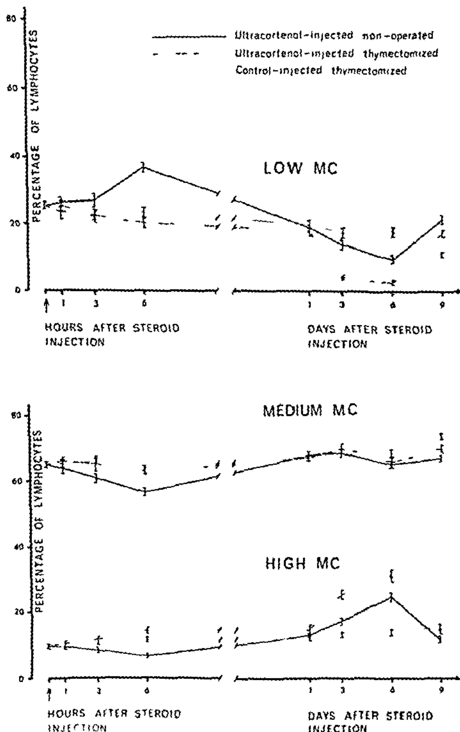

Ultracortenol-injected non-operated
Ultracortenol-injected thymectomized
Control-injected thymectomized
PERCENTAGE OF LYMPHOCYTES
LOW MC
MEDIUM MC
HIGH MC
HOURS AFTER STEROID INJECTION
DAYS AFTER STEROID INJECTION

Fig 5

solone induced involution and regeneration of the thymus in the guinea pig see *Gyllensten* 1962 *Ernstrom & Gyllensten* 1965)

At 3 and 6 hours after steroid injection pronounced granulocytosis appeared the number of granulocytes being 350 and 338 per cent of the control value (Table 1) In all the steroid treated animals an excess of granulocytes was found in carotid artery blood in comparison to thymic vein blood

TABLE 2

Thymic Emigration (Veno Arterious Difference in No of Cells/mm³ of Blood) of Small Lymphocytes Characterized by Low Mitochondrial Content in Controls and Steroid Treated Guinea Pigs A Decreased Output of Small Lymphocytes is Demonstrated 1 3 6 and 9 Days after Steroid Injection Mean Difference ± SE

	Controls	Interval after steroid injection 1 hour	3 hours	6 hours	1 day	3 days	6 days	9 days
No of emigrating small lymphocytes	629±167	413±168	320±181	548±164	135±109	111±36	139±23	156±106
t	3 77	2 46	1 77	3 34	1 24	3 08	6 04	1 47
df	15	9	11	11	9	10	10	10
p	<0 01	<0 05		<0 01		<0 05	<0 001	
Steroid induced decrease in no of emigrating small lymphocytes		216±237	309±246	81±234	494±199	518±171	490±169	473±198
t		0 91	1 26	0 35	2 48	3 03	2 90	2 39
df		24	26	26	24	25	25	25
p					<0 05	<0 01	<0 01	<0 05

At 6 hours after steroid injection the percentage of small thoracic duct lymphocytes (with low MC) was increased ($p<0.001$) and that of medium sized and large lymphocytes (with medium and high MC) correspondingly decreased ($p<0.001$ and $p<0.001$) At 3 and 6 days the percentage of small lymphocytes in the lymph was decreased ($p<0.001$ and $p<0.001$) and that of large lymphocytes markedly increased ($p<0.001$ and $p<0.001$) (Fig 3)

Thymectomized Animals

The total number of blood lymphocytes was decreased 1 hour after operation ($p<0.001$) the decrease comprising all subgroups of lympho-

Fig 3

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph of intact guinea pigs treated with a thymolytic steroid and of thymectomized guinea pigs treated with saline or steroid at different intervals after steroid injection and thymectomy Mean ±SE The arrow indicates injection of steroid and thymectomy

In the lymph of the thoracic duct thymectomy caused a decrease in the percentage of lymphocytes with low MC A minimum value was recorded 9 days after operation ($p<0\ 001$) The percentages of lymphocytes with medium and high MC were correspondingly increased (Fig 3)

Prednisolone Treated Thymectomized Animals

The total number of blood lymphocytes was reduced 3 hours after steroid injection in comparison to both non operated controls and thymectomized animals not given the steroid ($p<0\ 001$) Later after injection the total number did not differ significantly from the values in the control treated thymectomized animals at the corresponding time after operation (Table 3) The subpopulations of the blood lymphocytes changed markedly Thus the steroid caused an extremely pronounced deficiency of small blood lymphocytes in the thymectomized animals in comparison to the corresponding thymectomized controls (Fig 1) A highly significant reduction was noted as early as 3 and 6 hours after steroid injection At 1 day steroid treatment had not further potentiated the deficiency of small blood lymphocytes present after thymectomy without treatment At 3 and 6 days after thymectomy and steroid treatment however the steroid had produced a still more accentuated deficiency of small blood lymphocytes than that in the corresponding control treated thymectomized guinea pigs Then the smallest lymphocytes containing 0–5 mitochondria had practically disappeared from the arterial blood where they normally comprise 16 per cent of the lymphocytes At 9 days the small blood lymphocytes had reappeared At 1 day after steroid injection as well as later an increased number of medium sized and large blood lymphocytes was recorded in the steroid treated thymectomized animals in comparison to the corresponding thymectomized controls (Fig 1) This steroid effect was similar to that in the non operated guinea pigs

The granulocytosis observed 3 and 6 hours after thymectomy was not further potentiated by steroid treatment

The prednisolone induced increase in percentage of small thoracic duct lymphocytes occurring after 6 hours in the non operated guinea pigs did not take place in the thymectomized animals (Fig 3) The decrease in incidence of small lymphocytes (with low MC) 3 and 6 days after injection of prednisolone into the non operated animals also occurred in the thymectomized animals ($p<0\ 001$ and $p<0\ 001$ when compared to the corresponding thymectomized controls) and simultaneously the percentages of lymphocytes with high MC were increased ($p<0\ 001$ and $p<0\ 001$) The incidence of small lymphocytes in the lymph was 25 5 per cent in the non operated controls and at 6 days after treatment 9 4 per cent in the non operated prednisolone treated animals 17 5 per cent in the thymectomized controls and 2 5 per cent in the thymectomized prednisolone treated animals Thus

this effect of prednisolone was similar in non operated and thymectomized animals At this time the combined effect of thymectomy and prednisolone caused almost total depletion of the small lymphocytes in the lymph (Fig 3)

DISCUSSION

In a great number of normal guinea pigs the export of small lymphocytes from the thymus was found to be 629±167 cells per mm³ of blood passing through the organ The total blood flow through both thymus lobes in the young adult guinea pig is about 80 mm³ per minute (*Larsson* 1966a) This denotes a venous output of small lymphocytes from the thymus of 51×10^3 cells per minute or 74×10^6 cells per 24 hours The number of small lymphocytes per mm³ of arterial blood was 991±88 The blood volume can be estimated at 20 cm³ (*cf Ancill* 1956) representing 20×10^6 circulating small blood lymphocytes Thus the output of small lymphocytes from the thymus is sufficient to exchange the circulating small blood lymphocytes 3 7 times a day

A single injection of a thymolytic steroid (prednisolone) in the guinea pig reduced the export of small lymphocytes from the thymus to about 20 per cent of the normal value 1 3 6 and 9 days afterwards At the same time the number of circulating small lymphocytes was reduced It seems more probable that this reduction was caused by the decreased output of lymphocytes from the thymus than by intravasal lymphocytolysis as the latter effect of a steroid appears within 2 hours of its administration (*Dougherty* 1959) Autoradiographic analysis has demonstrated a delay of 1–3 days between the synthesis of DNA in the thymic lymphocytes and their appearance in the thymic vein blood in normal guinea pigs (*Larsson* 1966b) This delay is the same as the time between injection of the steroid and reduction of the output of lymphocytes from the thymus in the present investigation thus indicating an inhibition of DNA synthesis and cellular proliferation in the thymus as a possible cause of the decreased emigration of lymphocytes

During the regenerative phase at 6 and 9 days after injection of the steroid an import of medium sized and large lymphocytes into the thymus was demonstrated Simultaneously the number of circulating such cells was increased in blood and lymph Such an immigration of lymphocytes into the thymus was not demonstrable in normal guinea pigs Although the origin of the imported lymphocytes is not disclosed by the present investigation the report of *Ford & Micklem* (1963) indicates the bone marrow as a possible source of lymphocytes immigrating into an involuted and regenerating thymus

Thymectomy produced changes in the circulating lymphocyte populations similar to those seen in the guinea pigs with a steroid involuted thymus Thus a decreased number of circulating small lympho

cytes was demonstrated 1 3 and 6 days after operation whereas the large lymphocytes were increased in number Nine days after operation the number of small blood lymphocytes was almost normal In the present experiment no investigations were made later than 9 days after thymectomy It is true that the small lymphocytes were almost normal in number at this time It must nevertheless be pointed out that effects of thymectomy in newborn and young adult guinea pigs have been observed at later intervals after operation (*Ernström* 1965 a b *Ernström & Larsson* 1965)

Steroid treatment of the thymectomized guinea pigs caused a marked deficiency of small blood lymphocytes demonstrable statistically as early as 3 hours after prednisolone injection and most pronounced 3 and 6 days after it The absence of such an acute deficiency of small blood lymphocytes in the steroid treated guinea pigs with an intact thymus is probably due to the intact thymic export of small lymphocytes during the first 24 hours after steroid injection Experiments now in progress have even shown an increased blood flow through the thymus during this early phase thus perhaps indicating an increased outflow of thymic lymphocytes during the first hours after a steroid injection

In the thoracic duct lymph as well a drastic deficiency of small lymphocytes occurred in the animals which were both thymectomized and steroid treated The plausible explanation of this finding is that the small lymphocytes in the thoracic duct lymph originate essentially from the thymus at any rate during involution and regeneration of the lymphatic system after treatment with a steroid Since the lymphocyte population is subsequently normalized even in the thymectomized animals it is evident that other sources can compensate for the thymic contribution of small lymphocytes to the thoracic duct lymph

SUMMARY

Intact and thymectomized guinea pigs were treated with a single dose of prednisolone or saline The lymphocyte populations of carotid artery and thymic vein blood and of thoracic duct lymph were studied at different intervals after treatment The lymphocytes were divided into subpopulations classified by the cellular mitochondrial content which is correlated to the size of the lymphocyte Special attention was focused on the difference between the lymphocyte number in the blood from the carotid artery and from the thymic vein indicating a thymic import or export of cells The following observations were made

1 In the normal control animals there was a thymic export of 629± 167 small lymphocytes per mm³ of blood passing through the thymus This export was calculated to be sufficient to exchange the circulating small blood lymphocytes 3 7 times a day No significant import or

export of medium sized or large lymphocytes was demonstrated in the normal animals

2 At 1 3 6 and 9 days after a single injection of prednisolone the export of small lymphocytes from the thymus was decreased to 20 per cent of the normal value and the number and incidence of circulating small lymphocytes were reduced in the blood and lymph At 1 3 and 6 days after steroid injection the number and incidence of medium sized and large lymphocytes were increased in the blood and lymph and at 6 and 9 days after injection an import of such cells into the thymus was demonstrated

3 Thymectomy caused a decrease in the number and incidence of circulating small lymphocytes in the blood and lymph 1 3 and 6 days after operation Conversely the large lymphocytes were increased in number 3 6 and 9 days after operation

4 Prednisolone treatment of the thymectomized guinea pigs resulted in a deficiency of small blood lymphocytes highly significant as early as 3 hours after injection and most pronounced 3 and 6 days after it The smallest lymphocytes had then disappeared from the blood and lymph At 1 3 and 6 days after injection the medium sized and large lymphocytes were increased in number and incidence in the blood and lymph in the same way as in the non operated animals

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CHANGES IN BLOOD FLOW THROUGH THE THYMUS IN STEROID TREATED GUINEA PIGS WITH CALCULATION OF THYMIC EXPORT AND IMPORT OF LYMPHOCYTES



By

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The thymus of the guinea pig was shown by direct methods to be a potent contributor of lymphocytes to the circulating blood (*Ernström, Gyllensten & Larsson* 1965). This normal thymic export of cells via the efferent blood proved to consist mainly of small lymphocytes (*Ernström & Larsson* 1966).

The technique for direct measurement of the export of lymphocytes from the thymus offers new possibilities of studying the regulation of lymphocyte production and release. Among the factors known to influence this regulation are thyroid and steroid hormones. The thymic output of small lymphocytes was in fact greatly increased soon after administration of thyroid hormone (*Ernström & Larsson* 1965). Glucocorticosteroids cause characteristic involution followed by regeneration of the thymo-lymphatic system (*Selye* 1936, *Ingle* 1940, *Dougherty & White* 1945). These changes are most pronounced in the thymus, where well defined histological changes as well as inhibited cell formation have been described. The normal thymic export of 629±167 small lymphocytes per mm³ of blood passing through the thymus in the guinea pig was found to decrease to 20 per cent at 1, 3, 6 and 9 days after a single injection of prednisolone (*Ernström & Larsson* 1967).

For estimation of the lymphocyte producing capacity of the thymus in normal and prednisolone treated guinea pigs, it is of essential interest to measure the blood flow through the organ. A method for measuring the blood flow through the thymus was recently described (*Larsson* 1966). When the difference between the total number of lymphocytes per mm³ in thymic vein and carotid artery blood is known, as well as the blood flow through the thymus per minute, the total output of lymphocytes per minute can be calculated.

MATERIAL AND METHODS

Totally 72 male guinea pigs with an initial body weight of 281 ± 4 g (mean ± S.E.) were used. 23 of them as untreated controls. The rest received a single intraperitoneal injection of prednisolone (Ultracortenol® Ciba) in a dose of 10 mg/200 g b.w. The prednisolone treated animals were examined 3 and 6 hours and 1, 3, 6 and 9 days after the injection. They were anaesthetized with sodium Nembutal (0.3 ml/200 g b.w. of a 2.5 per cent solution i.p.). A femoral vein was exposed for administration of an anticoagulant (Heparin® Vitrum 3000 IU i.v. per 200 g b.w.). A 5 cm incision was made in the neck and the right or left internal jugular vein was exposed. The vein was ligated proximally and distally to the confluence of the thymic veins, as were all other small veins emptying into the internal jugular vein except the thymic veins (usually 2 or 3 in number). In some of the controls and some of the animals treated with prednisolone and dissected 6 hours afterwards the thymic veins were ligated and instead the veins from the submandibular gland were left open. A heparinized polyethylene catheter (outer diameter 1.2 mm, inner diameter 0.75 mm) could easily be introduced through a minor incision in the wall of the jugular vein and the blood flow through the thymus or the submandibular gland was led to a small container placed on a Mettler precision balance on which direct continuous readings could be made. A constant flow of 20–30 minutes was required. All dissections were made on a heating unit at 35° C. Immediately after dissection the wound was covered with liquid paraffin at 35° C *which was changed repeatedly during the operation. The blood container on the* Mettler balance was covered to prevent vaporization. The distal end of the catheter was placed so far below the proximal end that the capillary resistance of the catheter and the siphon effect equalized each other.

In most animals the right side was catheterized but the left side was also used. Both sides were catheterized in some of the controls. After dissection the animals were killed.

The results were analyzed statistically by means of Student's *t* test. The *p* values <0.05, <0.01 and <0.001 are denoted as almost significant, significant and highly significant, respectively.

RESULTS

No differences were recorded between the right and the left side.

In the control animals the blood flow through one thymic lobe was determined to be 41.0±4.9 mm³/minute (Table 1). The blood flow through the submandibular gland was 95.7±8.5 mm³/minute (Table 2).

TABLE 1

Changes In the Blood Flow Through the Right or Left Thymic Lobe at Different Intervals After a Single Injection of Prednisolone. Mean ± S.E.

Time after steroid treatment	No. of determinations	Body weight (g)	Thymic weight (mg)	Blood flow through right or left thymic lobe (mm³/min)
Controls	21	286±10	237±13	41.0±4.9
3	9	257±10	191±17	84.[illegible]±9.3
6 hours	8	266± 8	187±15	97.[illegible]±7.1
24	7	280±17	234±18	6[illegible].4±7.6
3	6	303± 8	206±15	57.5±9.6
6 days	8	309± 4	2[illegible]5±22	51.0±6.1
9	6	270±10	207±26	47.1±3.9

In the prednisolone treated animals the blood flow through the thymus was found to be highly significantly raised 3 hours after steroid injection (to 84 9±9 3 mm³/min) and after 6 hours the increase was still greater (to 97 6±7 1 mm³/min) At 24 hours the increase in blood flow was less pronounced (to 62 4±7 6 mm³/min $p<0\ 05$) Later no significant difference was noted in comparison to the controls and the values slowly decreased at 9 days being almost the same as those in the controls (Table 1) The blood flow through the submandibular gland was not found to be significantly changed in the steroid treated animals as compared to the controls (Table 2)

TABLE 2

Changes in the Blood Flow Through the Right or Left Submandibular Gland 6 Hours After a Single Injection of Prednisolone Mean ± S E

Time after steroid *treatment*	No of determi *nations*	Body weight *(g)*	Salivary gland weight (mg)	Blood flow through right or left submandi *bular gland* (mm³/min)
Controls	5	284±6	208±22	95 7±8 5
6 hours	5	276±5	204±11	85 4±7 0

DISCUSSION

The blood flow through the thymus was increased soon after treatment with prednisolone this increase being maximal 6 hours after steroid administration At this time no effect was recorded on the blood flow through a non lymphatic organ i e the submandibular gland This indicates that prednisolone may have a selective effect on the blood flow through the thymus (and perhaps other lymphoid organs) during its involution although an effect on the blood flow through other non lymphatic organs than the submandibular gland obviously cannot be ruled out

At 3 days and later after the steroid injection the blood flow through the thymus was increased but did not differ significantly from that in normal guinea pigs

Using the earlier found mean values of the cellular export of lymphocytes of different categories from the thymus of normal and steroid treated guinea pigs (*Ernstrom & Larsson* 1967) and combining them with the present data on the blood flow through the thymus the export of lymphocytes of different categories can be calculated (Table 3) In this way an initially increased output of small lymphocytes from the thymus is disclosed From 24 hours after steroid injection and onwards the venous output of small thymic lymphocytes decreased No export of large lymphocytes from the thymus was observed during

the experimental period On the contrary negative values were calculated These were most pronounced 6 and 9 days after injection of prednisolone indicating an immigration of large lymphocytes into the thymus during the regenerative period after treatment with steroid

TABLE 3

Calculation of the Thymic Output per Minute of Small Medium Sized and Large Lymphocytes at Different Intervals After a Single Injection of Prednisolone

Time after steroid treatment	No of Lymphocytes		
	Small	Medium sized	Large
Controls	51.6×10^3	10.0×10^3	-0.7×10^3
3	54.3×10^3	-0.8×10^3	-1.4×10^3
6 hours	107.0×10^3	7.8×10^3	-2.5×10^3
24	16.8×10^3	-13.3×10^3	-4.7×10^3
3	12.8×10^3	5.1×10^3	-9.4×10^3
6 days	14.2×10^3	-9.6×10^3	-18.9×10^3
9	14.7×10^3	-32.2×10^3	-11.3×10^3

SUMMARY

The blood flow through the thymus and the submandibular gland was measured in guinea pigs normally and during steroid induced involution and regeneration of the thymo lymphatic system

Steroid injection had a stimulating effect on the blood flow through the thymus which was maximally raised 6 hours after injection and gradually decreased to normal during the following week At 6 hours no effect was found on the blood flow through the submandibular gland

The present data on the blood flow through the thymus of normal and steroid treated guinea pigs were combined with previous results regarding the thymic veno arterial difference in number of lymphocytes of different categories An initial increase and later a pronounced decrease in the thymic export of small lymphocytes were revealed During the regenerative period of the thymus a net import of large lymphocytes into the organ was demonstrated

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EXPORT AND IMPORT OF ^{3}H THYMIDINE-LABELLED LYMPHOCYTES IN THE THYMUS OF NORMAL AND STEROID TREATED GUINEA PIGS

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Received 28 ii 67

The induction by corticosteroids of involution and regeneration of the thymo lymphatic system is well documented (*Selye* 1936 *Ingle* 1940 *Dougherty & White* 1945) Their mode of action especially that of the glucocorticosteroids on the thymo lymphatic organs is twofold first a lysis of cells during the first hours after administration (*Dougherty & White* 1943 1945 *Schrek* 1949 *Feldman* 1950) and secondly a decreased cell production occurring later and probably due to an inhibition of DNA synthesis (refs see *Ernstrom & Larsson* 1967)

These steroid induced changes are most marked in the thymus As this organ is a major contributor of the circulating small blood lymphocytes in the guinea pig (cf *Ernstrom Gyllensten & Larsson* 1965) it was of interest to investigate the thymic export during steroid induced involution and regeneration (see *Ernstrom & Larsson* 1967 *Larsson* 1967) Further information on the migration of lymphocytes into and from the thymus after steroid treament has now been gained by autoradiographic studies of newly formed cells both normally (*Larsson* 1966) and after steroid treatment (the present paper)

The autoradiographic technique has earlier been used to study the transport of cells within the thymo lymphatic tissue (*Cronkite et al* 1959 *Schooley et al* 1959 *Yoffey* 1960 *Diderholm* 1961 *Gowans* 1963 *Cannon & Whissler* 1965 *Lundmark & Fichtelius* 1965) The application of autoradiography to the blood of afferent and efferent vessels of the thymus for investigation of its cellular kinetics has not however been tested previously

MATERIAL AND METHODS

Totally 69 young male guinea pigs with an initial weight of [illegible] g (mean ± S E.) were used 10 of them as controls (The controls were th[illegible]me as those in an earlier experiment *Larsson* 1966) All the animals received a single intraperitoneal injection of tritiated thymidine (TRA 120 Thymidin (methyl T) The Radiochemical Centre Amersham England) in a dose of 0 5 m[illegible] b w 2 days before dissection The steroid treated animals were given a single [illegible] injection

of prednisolone (Ultracortenol® Ciba) in a dose of 50 mg/kg b w 1 3 and 6 hours and 1 3 6 and 9 days before dissection In each group 7 to 10 animals were examined The animals were anaesthetized with sodium Nembutal (25–50 mg/kg b w i p) The thymus was exposed and a thymic vein incised Blood samples were collected in heparinized blood pipettes (Heparin® Vitrum) for preparation of blood smears and for white cell counts in a Burker chamber The right carotid artery was incised close to the origin of the thymic artery and blood samples were taken for the same analysis as mentioned above The artery was then ligated Finally the thoracic duct was exposed at its confluence with the left subclavian and jugular veins mainly according to the technique of *Reinhardt* & *Yoffey* (1957) although the sternum was not incised The duct was punctured and lymph collected for preparation of smears

For counting of white cells in the Burker chamber 25 mm³ of blood was collected in a pipette and diluted with 475 mm³ of Toisson s solution containing methyl violet for staining of the white cells Totally 288 squares were counted for each determination

All the smears were allowed to dry in air and were then fixed in methanol for 5 minutes After drying the smears were covered with a thin film of celloidin (0 5 per cent celloidin in an alcohol ether solution) and dried again The slides were dipped into the photographic emulsion (Ilford Nuclear Research Emulsion K5 diluted in two parts of re distilled and filtered water dark room) and dried for 3 hours at 20 C Exposure proceeded for 1 month in absolute darkness at +4 C The slides were developed (Kodak D 19B) for 3 minutes fixed for 15 minutes and washed in running water for at least 20 minutes While still wet the slides with the blood and lymph smears were stained in Giemsa solution (200 drops in 100 ml of re distilled and filtered water) for 60 minutes Finally the smears were dipped into alcohol of 70 90 95 and 100 per cent covered with celloidin and a cover slip put on

The cell counts were performed in a light microscope at a magnification of 600 to 1000 X The background labelling was as a rule scanty A minimum load of 3 grains/cell was required for the cell to be regarded as labelled Altogether 100 to 400 cells were counted in each smear The percentage of labelled and unlabelled lymphocytes monocytes and granulocytes was registered The percentage of labelled lymphocytes was calculated from the percentage of total lymphocytes The absolute numbers of labelled lympho ytes were calculated from the total number of white cells and the percentage of labelled lymphocytes

The results were analyzed statistically by Student s *t* test The *p* values <0.05 <0.01 and <0.001 are denoted as almost significant significant and highly significant respectively The comparisons between the number of labelled lymphocytes in thymic vein and carotid artery blood were performed by statistical analyses of the differences in the individual animals

RESULTS

The percentage of labelled lymphocytes in thymic vein and carotid artery blood was raised 3 and 6 hours after steroid injection (Fig 1) the increase being highly significant in the arterial blood Thus the normally lower percentage of labelled lymphocytes in carotid artery blood had reached the values of the thymic vein blood after 3 hours One day after steroid treatment a parallel decrease in the percentage was noted in both thymic vein ($p < 0.01$) and carotid artery blood ($p < 0.05$) compared with the values at 6 hours The percentage of labelled lymphocytes in the afferent thymic blood had then returned to the normal level whereas that in efferent thymic blood was decreased below it ($p < 0.01$) Three days after steroid administration the percentage of labelled lymphocytes was further decreased in both thymic vein and carotid artery blood the values being almost the same At 6

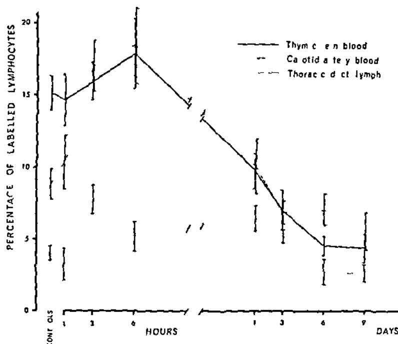


Fig 1

Changes in the percentage of labelled lymphocytes in thymic vein blood carotic artery blood and thoracic duct lymph at different intervals after a single injection of prednisolone Mean ± S E

and 9 days after injection the percentage of labelled lymphocytes in carotid artery blood exceeded the corresponding values in thymic vein blood although the differences were not significant At these times the percentages in thymic vein blood were markedly lower than in the controls ($p < 0\,001$ and $p < 0\,001$ respectively) amounting to only 30 per cent of the normal value

During the experimental period the absolute number of labelled lymphocytes (Fig 2) showed similar variations to those in the per centage values This applied to both thymic vein and carotid artery blood *Thus the maximal number of labelled lymphocytes was found* 3 hours after injection of steroid the increase being highly significant in carotid artery blood The fewest labelled lymphocytes were recorded 3 6 and 9 days after steroid treatment At 6 and 9 days the number in carotid artery blood exceeded that in thymic vein blood this difference being almost significant on day 6 The number of labelled lymphocytes in thymic vein blood was then only 30 per cent of the normal value

The veno arterial difference in labelled lymphocytes per mm^3 of blood (Fig 3) was highly significant in the controls and the animals investigated 1 hour after steroid administration not significant 3 and 6 hours after injection and significant 1 day after At 3 days no differ ence was present Six days after steroid treatment the difference be

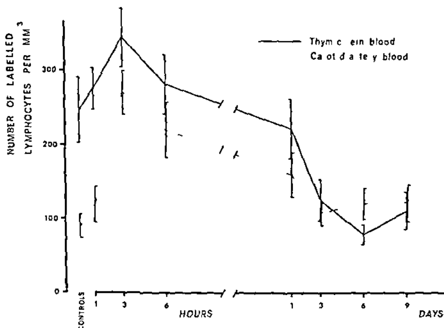


Fig 2

Changes in the number of labelled lymphocytes per mm³ in thymic vein and carotid artery blood at different intervals after a single injection of prednisolone Mean ± S E.

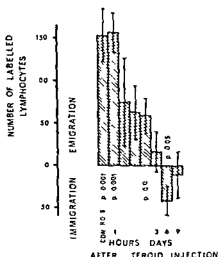


Fig 3

Changes in the thymi veno arterial differ n in number of labelled l mphoc tes per mm³ at different intervals after a single injection of prednisolone Mean ± S E.

came negative (i e, more labelled lymphocytes in carotid artery blood than in thymic vein blood $p < 0.05$) indicating a net immigration of labelled cells into the thymus At 9 days no veno arterial difference was observed

The percentage of labelled lymphocytes in thoracic duct lymph (Fig 1) was increased 3 hours and 1 day after steroid injection ($p < 0.01$ $p < 0.05$) At 6 and 9 days after steroid treatment the percentage was decreased below the normal level although not significantly

DISCUSSION

The normal export of small lymphocytes from the thymus was recently determined to be 629 ± 167 cells per mm³ of blood passing through the organ (*Ernstrom & Larsson* 1967) A single injection of tritiated thymidine does not label more than about 20 per cent of the small lymphocytes in the thymic cortex and in the present study the veno arterial difference in labelled lymphocytes was 153 ± 31 cells per mm³ of blood

The glucocorticosteroids are known to cause on one hand lysis of the already formed cells in the thymic cortex on the other hand inhibition of cell proliferation there probably by interfering in DNA synthesis (for references see *Gyllensten* 1962 *Ernström & Gyllensten* 1965) The present study indicates the occurrence of an increased delivery of newly formed lymphocytes to the blood during the first few hours after a single injection of prednisolone (Fig 2) During this early period after steroid injection the blood flow through the thymus is increased (*Larsson* 1967) This increased blood flow results in a raised output of lymphocytes from the thymus (*Larsson* 1967) thus explaining the high incidence of labelled lymphocytes in the blood noted 3 hours after steroid injection Since 1–3 days elapse between DNA synthesis and migration of the thymic lymphocytes from the organ (*Larsson* 1966) the decreased production of thymocytes is not reflected until about 3 days after steroid injection The number of labelled lymphocytes per mm³ in thymic vein and carotid artery blood was then almost identical possibly due to inhibition of the export of newly formed lymphocytes from the thymus Later i e 6 days after steroid injection there was a negative veno arterial difference in number of labelled lymphocytes per mm³ indicating a net import of newly formed cells into the thymus This is in agreement with recent findings i e that 6 and 9 days after a single steroid injection a net immigration of medium sized and large lymphocytes into the thymus occurs (*Ernstrom & Larsson* 1967) These cells may originate in the bone marrow (*Ford & Micklem* 1963) Nine days after steroid injection no thymic veno arterial difference was present and thus no export or import of newly formed lymphocytes in the thymus was established

In the thoracic duct lymph a significant increase in the percentage

of labelled cells was recorded 3 hours after injection Although this may have been due to passage of small thymic lymphocytes from the blood to the lymph during the early period after steroid injection the newly formed lymphocytes may also have been of extra thymic origin Later during involution and regeneration following steroid treatment the percentage of labelled lymph cells did not differ significantly from the control value

TABLE 1

Calculation of the Thymic Output of Labelled Lymphocytes per Minute at Different Intervals After a Single Injection of Prednisolone

Time after steroid injection	No of animals	Body weight (g)	Thymic export or import of lymphocytes/min
Controls	9	241±7	6?73
3	7	248±11	6378
6 Hours	6	250±9	6149
24	10	269±9	368?
3	10	259±4	978
6 Days	10	249±3	—?14?
9	8	?62±2	—518

If the present values of veno arterial differences per mm³ of blood are combined with earlier data on the blood flow through the thymus after steroid treatment (*Larsson* 1967) the total export and/or import of cells in the thymus per minute can be calculated (Table 1) In the controls and soon after steroid administration an output of labelled lymphocytes occurs Later during regeneration of the thymus at net import of labelled lymphocytes is found This is in accordance with earlier observations (*Ernstrom & Larsson* 1967 *Larsson* 1967)

Theoretically a steroid induced change in the re utilization of thymidine and of the size of the thymidine pool in the thymus may have influenced the appearance of labelled lymphocytes during the latter part of the experiment when ³H thymidine was given after the steroid A decreased output of lymphocytes from the thymus after steroid administration is however in agreement with earlier findings achieved by other methods (*Frnstrom & Larsson* 1967)

SUMMARY

Normal and steroid treated guinea pigs were investigated 2 days after an intraperitoneal injection of tritium labelled thymidine The percentage and total number of labelled lymphocytes in afferent and efferent thym c blood and i thor i duct lymph were registered The steroid treated animals w e ex i mined at different intervals after steroid injectio n The foll in i ndings were made

1 The percentage of labelled lymphocytes in thymic vein and carotid artery blood was increased 3 and 6 hours after steroid treatment Later the percentage decreased so that 3 6 and 9 days *after injection* it was markedly lower than in the controls

2 The absolute number of labelled lymphocytes per mm^3 in thymic vein and carotid artery blood varied similarly to the corresponding percentages Thus the number of labelled lymphocytes was greatest 3 hours after steroid injection and smallest during the regenerative period of the thymus

3 The veno arterial difference in number of labelled lymphocytes per mm^3 of blood was decreased soon after steroid injection Six days afterwards during the regenerative phase of the thymus there was a net import of newly formed labelled lymphocytes into the thymus

4 The percentage of labelled lymphocytes in thoracic duct lymph was increased soon after steroid treatment During the regenerative period the percentage was decreased

5 The present values of thymic veno-arterial differences in labelled lymphocytes per mm^3 of blood were combined with earlier data on the blood flow through the thymus after steroid treatment An export of labelled cells was calculated soon after injection whereas during the regenerative period on the contrary an import occurred

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but preliminary experiments with retinoic acid indicated that much smaller doses were sufficient to induce teratogenesis. Mother animals were sacrificed on the morning of day 18 of pregnancy for mice and day 20 for rats. The fetuses were examined for external malformations either fresh or after Bouin's fixation; for visualization of skeletal defects they were cleared in potassium hydroxide and stained with alizarin red.

RESULTS

The data in Table 1 compare the effects produced by retinoic acid and retinyl acetate treatments on the reproductive performance of A/Jax mice. Three dose levels of these compounds could be compared on the basis of obtainability of both a similar range of subsequent embryonic death and resorption and a comparable incidence of fetal malformations.

TABLE 1

Comparison of Effects of Retinoic Acid and Retinyl Acetate on the Reproduction and Malformations in Mice. Treatment Was Given only on a Single Day

Agent	Dose/20gm bodyweight mg	Dose/20gm bodyweight I U	Days of treatment	Number of animals	Number of implantation sites	% Embryos resorbed	% Survivors malformed
Retinoic acid	4	1000	8	5	42	100	–
			9	2	16	100	–
			10	4	30	97	100
Retinyl acetate	40	40 000	9	4 (2 died)	18	94	100
Retinoic acid	2	500	9	7	50	70	100
			9	5	37	32	44
Retinyl acetate	20	20 000	8	11	93	75	17
			9	14 (4 died)	80	36	47
Retinoic acid	1	250	8	5	41	24	45
			9	5	37	16	43
Retinyl acetate	10	10 000	8	2	21	29	20
			9	3	26	8	50
			10	4	28	25	33

Only one fetus survived which had severe malformations.

Administration of 4 mg of retinoic acid or 1000 I U according to *van Dorp & Arens* (1946) on any of 8, 9 or 10 days of gestation was very toxic to the fetuses. All but 1 out of a total of 88 implantation sites resorbed in response to this treatment. The lone survivor was very severely malformed with a shortened trunk and extreme exophthalmos and had several of the defects to be mentioned later for the fetuses treated with smaller doses. The dose of retinyl acetate having a comparable lethal effect on the fetuses was in the range of 40 mg (40 000

i u) This treatment was toxic for the pregnant animal as well two of the 4 treated mothers died and the remaining animals yielded only 1 living fetus out of 18 implantation sites This fetus also had all the defects which are described below

Administration of 2 mg retinoic acid (500 i u) on a single day was a very teratogenic dose without having an extreme toxic effect on the fetuses For some unknown reason two batches of animals treated two months apart first in January 66 and the second in March 66 gave quite different responses Seven animals in the first batch treated on day 9 yielded 15 living fetuses all very severely malformed All of these fetuses looked alike and the abnormalities they had could be described as comprising a syndrome These fetuses had markedly shortened trunk extreme exophthalmia microcephalia astomia rudimentary or missing mandible tail stump no genital opening malformed sternum and ribs missing vertebrae and some degree of agenesis of the hind limb Spina bifida occulta in the sacral region occurred in 9 out of 15 The second batch of 5 animals again treated on day 9 yielded 25 living fetuses (a resorption rate of 32 per cent compared to 70 per cent in the first batch) with no typical syndrome as described above Eleven fetuses among these had abnormalities (44 per cent) such as cleft palate and some rib malformations The dose level of retinyl acetate which gave a comparable pattern of malformations was again toxic for the mother where 4 out of 14 treated animals died A dose of 20 000 i u of retinyl acetate on day 9 produced 36 per cent resorption Twenty four out of 51 live fetuses obtained had various malformations 8 of these had a typical syndrome described for retinoic acid all with spina bifida occulta Fifteen others had cleft palate these fetuses also had one or more of such malformations as stumpy tail malformed ribs hind limb agenesis and exophthalmia One fetus had only rib abnormalities 20 000 i u retinyl acetate on day 8 produced quite high resorption rate (75 per cent) but a low rate of malformations only 4 out of 23 fetuses had cleft palate and some rib and sternum malformations One female treated with 500 i u of retinoic acid on day 8 died

With 1 mg retinoic acid (250 i u) although a slightly higher resorption rate (24 per cent) was obtained on day 8 than what it was on day 9 (16 per cent) the per cent incidence of malformations in the fetuses was identical on these two days Major abnormality encountered was cleft palate it occurred in 13 out of 14 fetuses on the earlier day and 10 out of 13 on the later Many of these fetuses showed dermal tabs on the face Other abnormalities observed were microphthalmia and anophthalmia There were no malformations of the type observed with higher doses The results with this lower dose of retinoic acid compare well with those obtained with 10 mg (10 000 i u) of retinyl acetate Most commonly observed abnormality here was also cleft palate with facial dermal tabs there were some eye malformations as well as a few skeletal abnormalities in the tail sternum & ribs

TABLE 2

Comparison of Effects of Retinoic Acid and Retinyl Acetate on the Reproduction and Malformations in Rat Indicated Dose Was Given Daily for 3 Days

Agent	Dose mg	Dose i u	Days of treatment	Number of animals	Number of implantation sites	% Embryos resorbed	% Survivors malformed
Retinoic acid	5	1250	9 10 & 11	2	19	95	100
	10	2500		12	112	89	100
Retinyl acetate§	60	60 000		13	114	54	80
Retinoic acid	5	1250	10 11 & 12	4	37	49	5
	10	2500		4	37	8	100
	20	5000		1	10	70	100
Retinyl acetate§	60	60 000		11	112	[illegible]	83

Only one fetus survived which had severe malformations
§ Data from *Kochhar & Johnson* (1965)

Retinoic acid was found quite a potent teratogen also in the rat Table 2 summarises this data The treatment period of 9–11 gestational days was very toxic for the embryos One live fetus out of 19 implants obtained after 5 mg daily (1250 i u) treatment was severely malformed It had anencephaly and cleft palate With a daily dose of 10 mg (2500 i u) 12 fetuses survived out of 112 implants 10 of these had sacral spina bifida 1 had exencephaly and spina bifida and 1 had sirenomelia Many had a stumpy tail and some had cleft palate as well Comparing these results with those obtained with retinyl acetate administered over the similar treatment period a dose of 60 000 i u produced a resorption rate of 54 per cent and 80 per cent of the survivors were malformed with such deformities as exencephaly (15 per cent) cleft palate (80 per cent) and eye malformations (58 per cent)

The treatment period of 10–12 days was much less lethal but equally teratogenic except with the lower dose With 5 mg daily dose only 1 out of 19 living fetuses obtained was malformed it was much smaller than its other normal litter mates it weighed 1 7 gms in contrast to a normal average of 4 0 gms It also had marked exophthalmos After a daily treatment with 10 mg retinoic acid only 3 out of 37 implants were resorbed all the remaining 34 had such malformation as microstomia open eyes spina bifida short tail many of these with cleft palate One female given 20 mg retinoic acid daily had 10 implants out of which 7 resorbed the three living had exophthalmia 2 of them with spina bifida & micrognathia 1 also with gastroschisis and 1 with agnathia Correspondingly a treatment with 60 000 i u of retinyl acetate during this

gestational period also showed lower resorption rate of 22 per cent Eighty three per cent of the survivors were abnormal having cleft palate and eye malformations

DISCUSSION

These results show that retinoic acid is teratogenic at amounts much smaller than what are essential for retinyl acetate to produce malformations Since biological activities of the two compounds in restoring normal growth in vitamin A deficient animals are reported to be approximately equal (*Murray* 1962 *de Man et al* 1961) in order to account for the quantitative differences in their teratogenic activities one must seek some other differences in their biochemical and physiological properties It has been found (*Thompson & Pitt* 1961 *Thompson* 1965) that retinoic acid is much more potent than retinol in inducing signs of hypervitaminosis A in rats Also its toxicity is much in excess of that of retinol as revealed in organ culture experiments on Xenopus laevis (*Weissmann Bell & Thomas* 1963) and chick limb rudiments (*Fell Dingle & Webb* 1962) and in incubating chicken eggs (*Thompson et al* 1965) It is quite probable that the higher potency of retinoic acid as encountered in these experiments also accounts for its increased teratogenic activity

Some of the previous workers investigating retinoic acid for its ability to ameliorate lesions of vitamin A deficient animals have also observed it to produce effects on their reproductive functions which can be considered to be of a teratological nature *Thompson et al* (1964) administered retinoic acid to previously vitamin A deficient pregnant rats in amounts of 1 mg per rat per day throughout pregnancy No living fetus was obtained they were found to be in various stages of resorption This embryotoxic effect was however ascribed to the conditions of vitamin A deficiency still prevailing in the mother which retinoic acid had failed to prevent Adequate support to this fact was given by control experiments where deficient animals when given plentiful supply of retinol along with retinoic acid cast normal litters Nevertheless the fact remains that on the basis of observations reported here the amounts of retinoic acid administered to animals by *Thompson et al* (1964) were sufficient to have caused embryonic death and resorption directly Parallel findings were also reported in the chick (*Thompson et al* 1965) where eggs from vitamin A deficient hens maintained with retinoic acid showed abnormal development of the embryo

The types of malformations observed with the two agents for the most part were similar both in rat and the mouse in the rat however *spina bifida occurred only after retinoic acid and not after retinyl acetate* This may not be indicative of any significant difference in teratogenic activity since spina bifida has been observed earlier in rats after retinyl acetate (*Cohlan* 1953 *Giroud & Martinet* 1956) However the

incidence reported was quite low. Increased incidence of spina bifida may possibly be due to the ability of retinoic acid to produce conditions of severe hypervitaminosis A not possible to achieve with the amounts of retinyl acetate employed in these experiments (*Kochhar & Johnson* 1965).

For general insights into the mechanism of action of teratogenic agents the use of hypervitaminosis A holds great interest because some of the previously unknown metabolic involvements of vitamin A are gradually coming to light. From a review by *Wolf* (1962) it is apparent that vitamin A has a profound effect on such tissues as epithelium and cartilage which are characterized by the presence in them of considerable amounts of mucopolysaccharides. Synthesis of mucopolysaccharides by mucus secreting cells of rat colon has in fact been shown to be vitamin A dependant (*Wolf & Varandani* 1960). The well known effect of vitamin A on the cartilage in dissolution of mucopolysaccharides from its intercellular matrix is mediated by what has now come to be known as a generalized effect that the vitamin has on the membranes of a number of cells and intracellular organelles (*Dingle & Lucy* 1965).

Some of these biochemical effects of vitamin A can be experimentally investigated for their possible role in teratogenesis. One study directed to this end (*Kochhar & Johnson* 1965) has shown alterations in the metabolism of sulfated mucopolysaccharides of mesenchymal tissue and cartilage of malformed embryos following maternal treatment with retinyl acetate.

One shortcoming encountered previously in the use of retinyl esters as teratogenic agents is that once administered they remain in the mother's body too long; consequent prolonged exposure of the developing embryo to them interferes in attempts to critically analyze the embryonic stage susceptible to their teratogenic influence. Such an approach may become feasible now since retinoic acid, even after administration of high doses, rapidly disappears from tissues of animals (*Arens & van Dorp* 1946, *Sharman* 1949). Because of this advantage and others mentioned above retinoic acid may prove to be an agent of choice for further studies on hypervitaminosis A induced teratogenesis.

SUMMARY

All trans retinoic acid when administered to pregnant rats and mice produces congenital malformations in their fetuses. This teratogenic response is elicited with a much smaller dose and greater severity than previously reported for other vitamin A compounds. On the basis of biological activity retinoic acid is 40 times as active as retinyl acetate in producing congenital malformations in the mice. Under the conditions employed in the present experiments only retinoic acid produces spina bifida in the rat. It is suggested that the greater teratogenic activity of retinoic acid may be related with its higher toxicity noticed

in earlier studies It is hoped that comparative study of teratogenic activities of two or more closely related compounds such as retinoic acid and retinyl acetate may eventually lead to understanding of the mechanism of action

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COMPLEMENT FIXING LIVER ANTIBODIES

3 *Their Serological Relationship to Thyroid Auto antibodies and Rheumatoid Factors in Patients with Chronic Hepatitis and in Normal Blood Donors*

By

G Norup

Received 22 xii 66

The demonstration of serum substances reacting *in vitro* with extracts from various tissues in patients with cirrhosis of the liver has led to the assumption that immunological processes might play a role in the pathogenesis of chronic hepatitis

Non species and non organ specific antibodies against cytoplasmic antigens in liver cells and bile ductular epithelium have been observed by complement fixation using suspensions of various human and animal tissues as antigen (*Bjorneboe & Krag* 1947 *Gajdusek* 1958 *Deicher et al* 1960 *Gokcen* 1962 *Pasnick et al* 1962 *Walker et al* 1965 *Norup* 1967) and by the immunofluorescent antibody technique using cryostat se tions of human and animal liver kidney thyroid gland and smooth muscle as antigen (*Hunter et al* 1960 *Paronetto et al* 1961 1964 *Bumbalo et al* 1962 *Johnson et al* 196 *Whittingham et al* 1966 *Doniach et al* 1966) Cases of active chronic hepatitis associated with a positive LE cell test (*lupoid hepatitis*) have been reported by several investigators (*Joske & King* 19 5 *MacKay et al* 1956 *Anderssen & Skjæggestad* 1962 *Ma ka y & Wood* 1962) Antibodies to different anti genic components of cell nuclei have been observed with varying frequency in sera from patients with chronic hepatitis using the complement fixation technique (*Asherson* 1959 *Deicher et al* 1960) the immunofluorescent technique (*Calabresi & Greenberg* 1960 *Weir et al* 1961 *Holborow et al* 1963 *Bouchier et al* 1964 *Faber & Elling* 1965 *Doniach et al* 1966) and the anti gammaglobulin consumption technique (*Russel et al* 1957 *Finke* 1965)

In all the studies referred to abo e positive reactions occurred most frequently in patients with posthepatitic (postviral postnecrotic juvenile) cryptogenic (active chronic hepatitis lupoid hepat tis plasma cell hepatitis) and primary biliary cir rhosis whilst in patients with alcoholic or second ry b liary cirrhosis the antil dy findings did not differ from those ob erved in materials of healthy individuals (*MacKay & Larkin* 1958 *Gokcen* 196 *Norup* 1967)

Antibody to thyroglobulin which is generall assumed to be organ spe ific and which occurs in 85 90 per cent of era from patients with Hashimoto s th roiditis (*Roitt & Doniach* 1958 *Hjort* 1963) has been ob erved with va rying frequenc in patients with chronic hepatit s and in healthy individuals (Table 1) In all the studies referre l to in this table no relationship between the incidence of po itive T R C tests and acti logical type of chronic hepatitis was found.

Rheumatoi l fa t rs a complex of macr gl bulins ccurring in sera from 5 85 per cent of the patient with rheumat i l arth tis and from 0–8 per cent of n rmal individuals (*Z ff* 1957 *W ablad* 1960b *Seraf ni et al* 1964) ha e been found with

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varying frequency in patients with hepatic cirrhosis in some extent depending on the technique employed In Table 2 the results obtained with the latex fixation test in chronic hepatitis are summarized It appears that a positive latex fixation test was frequently found in patients with chronic hepatitis whilst a negative test was observed in most of the cases with biliary cirrhosis

Using the sensitized sheep cell test (Wåler Rose reaction) rheumatoid factor has been reported in 5–20 per cent of sera from patients with hepatic cirrhosis (*Harter & Diekgiesser* 1953 *Winblad* 1960b *Waldenström et al* 1964) Studies in which the sensitized sheep cell test has been carried out in parallel with one or more of the so called F II tests (the latex fixation test of *Singer & Plot* (1956) the bentonite flocculation test of *Bozicevich et al* (1958) the acryl fixation test of *Winblad* (1960a)) on the same group of sera have shown that the sensitized sheep cell test was positive less frequently than any of the F II tests (*Winblad* 1960b *Holley et al* 1961 *Bouchier et al* 1964)

TABLE 1

Results of Tanned Red Cell (T R C) Test in Chronic Hepatitis and Healthy Individuals

Authors		Chronic Hepatitis		Healthy Individuals	
		Total Tested	No of +ve	Total Tested	No of +ve
Hackett et al	1960a	24	[illegible]	10[illegible]	9
Skanse & Nilsson	1961	33	1	250	10
Mackay & Wood	1962	5[illegible]	10	168	7
Bouchier et al	1964	104	10		
Doniach et al	1966	158	41	158§	2[illegible]

Results of immunofluorescent test for colloid antibody are included

§ Matched for sex and age with the patients

TABLE 2

Results of Latex fixation Tests Carried out on Sera from Patients with Hepatic Cirrhosis of Various Types

Authors		Chronic Hepatitis		Biliary Cirrhosis Secondary		Primary	
		Total Tested	No of +ve	Total Tested	No of +ve	Total Tested	No of +ve
Dresner & Trombly	1959	69	17	7	2		
Howell et al	1960	25	9	8			
Atwater & Jacox	1963	71	41	13	10		
Caplan	1963	[illegible]9	23	26	1		
Butler & Paton	1964	[illegible]1	15	17	1		
Walker et al	1965			[illegible]1	4	3[illegible]	9

Chiefly of the alcoholic type (Laennec s cirrhosis)

Polish & Muschel (196[illegible]) examined sera from 29 patients with cirrhosis of the liver (10 with postnecrotic 6 with chronic hepatitis and 13 with alcoholic cirrhosis or fatty liver) for rheumatoid factor using the bentonite flocculation test and for complement fixing antibodies to suspensions of rabbit liver and calf thymus as well as to nucleoprotein derived from calf thymus Of the 16 cases with postnecrotic cirrhosis and chronic hepatitis rheumatoid factor was found in 7 and complement fixing antibodies to liver and/or thymus tissue in 5 However in no case were rheumatoid factor and complement fixing antibody found to be present in the same

serum None of the ases with alcoholic cirrhosis had rheumatoid factor although complement fixing tissue antibody was observed in the sera from 4 of them

Whilst several studies on the frequency of circulating tissue antibodies and rheumatoid factors in materials of patients with chronic hepatitis have been re ported only one of the investigations referred to above gives any information about the frequency with which the different antibodies occur in serum from the same patient

Since it has been shown that circulating complement fixing tissue antibodies are just as characteristic of disseminated lupus erythema tosus as are rheumatoid factors of rheumatoid arthritis and antibody to thyroglobulin of Hashimoto s thyroiditis it has been considered of interest to determine the frequency of the occurrence of the above mentioned antibodies in a material of patients with chronic hepatitis and to correlate the incidence of these antibodies in the individual patient

MATERIAL AND METHODS

Sera from 135 patients with different types of chronic hepatitis were studied The distribution of the patients with regard to sex and age and to the four aetiological types (posthepatitic cryptogenic alcoholic and biliary) of cirrhosis has been given in a previous paper (Vorup 1967) In addition a variable number of sera from 194 normal blood donors selected as previously described (Vorup 1965) were examined

Rheumatoid factors in the sera were demonstrated by 1) the *latex fixation test* 2) the *sensitized sheep cell test* (Wåler Rose reaction) and 3) the *streptococcal agglutination test*

1) The *latex fixation test* (Hyland RA test) was performed according to the manu facturer s instruction The titre of a serum was read as the reciprocal of the highest dilution causing macroscopically visible agglutination of the latex particles Titres ≧ 20 were considered positive

2) The *sensitized sheep cell (S S C) test* was performed by a modification of the technique described by *Heller et al* (1949 1952) 1

Preparation of *anti sheep erythrocyte rabbit serum* (amboceptor) Sheep cells for injection were washed three times in saline and a 50 per cent suspension was pre pared The rabbits were immunized according to the following inoculation schedule two series each of 5 injections of 0 5 ml were given on alternate days with an interval of 7 days 6 days after the last injection the agglutination titre for sheep erythrocytes was determined in ear blood If the agglutination titre was suitable (1 500 or more) the rabbit was bled After inactivation (56 C for 30 min) the rabbit serum was distributed on small tubes in amounts of two ml and stored at —20 C No significant decrease in haemagglutinating ability was observed after 5–6 years storage at this temperature The amboceptor could be thawed and frozen repeatedly (15–20 times) without appreciable titer fall

Titration of anti sheep erythrocyte rabbit serum Dilutions of serum ranging from 1 100 to 1 2400 were made up in volumes of 0 5 ml in Wassermann tubes and 0 5 ml of a one per cent suspension of normal sheep cell was added The tubes were incubated in a water bath at 37 C for 60 mins and subsequently at room temper ature (20 -22 C) for 60 mins whereafter reading was performed The reciprocal of the highest initial dilution of amboceptor showing macroscopically visible agglutina tion of the sheep cells was designated the minimum agglutinating dose (MAD)

Sensitization of sheep erythrocytes Sheep cells from defibrinated blood were washed three times in saline whereafter a two per cent suspension was prepared Equal volumes of this suspension and anti sheep cell rabbit serum diluted to 4 MAD per ml were mixed and incubated in water bath at 37 C for 20 mins The mixture was stored at 4 C until required A new portion of sensitized cells was prepared for daily use

Performance of the test After inactivation at 56 C for 30 mins one ml serum was

1 The routine technique used in the Streptococcal Department of this Institute

absorbed with 0 25 ml packed sheep cells twice at room temperature for 40 mins The serum was diluted two fold in saline in volumes of 0 2 ml starting with 1 5 whereafter 0 2 ml of a one per cent suspension of sensitized cells was added To a control series comprising the three lowest dilutions of serum 0 2 ml of a one per cent suspension of normal sheep cells was added After shaking the tubes were incubated in a water bath at 37 C for 60 mins and at room temperature for 60 mins where after reading was performed The agglutination pattern was graded with the naked eye as follows 2 compact agglutinate or 2–6 large clumps 1 medium sized or small clumps 0 no agglutination The titre of a serum was read as the reciprocal of the highest dilution giving a 1 reaction Titres ≧ 40 were considered positive

3) *Streptococcal agglutination test* (SAT) was performed according to the routine technique used in the Streptococcal Department of this Institute (*Kalbak* 1948) 0 5 ml of a living culture of haemolytic streptococci SF 130 (Group A type 1) was added to 0 5 ml of inactivated serum diluted two fold starting with 1 20 After incubation in a water bath at 52 C for two hours the agglutination patterns were read with the naked eye and graded by numbers from 4–0 4 representing a large coherent disc 3 2 and 1 coarse medium sized and small clumps respectively and 0 no agglutination The titre of a serum was read as the reciprocal of the highest dilution giving a 1 reaction Titres ≧ 20 (reaction 2) were considered positive

Tissue antibodies in the sera were demonstrated by 1) *the tanned red cell test* and 2) the *complement fixation test*

1) *Tanned red cell (TRC) test* was mainly performed by the modification of *Boyden* s haemagglutination technique (*Boyden* 1951) described by *Fulthorpe et al* (1961)

Thyroglobulin was prepared from normal thyroid tissue (necropsy) by the method of *Derrien et al* (1948)

Formalin treatment of sheep erythrocytes was performed by a slight modification of the technique described by *Weinbach* (1958) Sheep blood in modified *Alsever* s solution (*Bukant et al* 1946) was washed three times in saline and then suspended in phosphate buffer pH 7 2 to give a 6 per cent suspension Equal parts of this suspension and formalin (3 vol/per cent formaldehyde) were mixed and incubated in a water bath at 37 C for 18 hours After washing three times in phosphate buffer a three per cent suspension of formalin treated cells was prepared

Tannin treatment of the cells was carried out by mixing equal parts of a three per cent suspension of formalin treated cells and a tannic acid solution 1 20 000 After incubating in a water bath at 37 C for 15 mins the cells were washed three times in phosphate buffer and then suspended to produce a three per cent suspension

Coating with thyroglobulin To a 0 2 per cent (w/v) solution of human thyro globulin was added a three per cent suspension of formalin and tannin treated cells in equal parts The mixture was incubated in a water bath at 37 C for 30 mins and after washing twice in phosphate buffer and once in borate succinate buffer pH 7 5 the thyroglobulin coated cells were suspended in a borate succinate buffer which contained normal rabbit serum in a concentration of one per cent to produce a one per cent suspension of cells

Performance of test 0 1 ml serum to be tested was absorbed with 0 4 ml of a one per cent suspension of formalin and tannin treated cells at room temperature for 15 mins and after centrifuging titrated in 10 fold dilutions starting with 1 5 In a haemagglutination tray 0 1 ml dilution of serum was mixed with 0 1 ml of a one per cent suspension of thyroglobulin coated cells As a control 0 1 ml serum dilution 1 5 was mixed with 0 1 ml of a one per cent suspension of formalin and tannin treated cells The haemagglutination tray was gently shaken and left at room temperature for 4 hours after which reading was performed The agglutination patterns were evaluated with the naked eye and graded as follows 3+ thin carpet of cells covering the bottom and walls of the cup 2+ smooth mat of cells covering a smaller area of the walls 1+ thicker mat of cells with a narrow ring around the edge and – small disc of cells with a heavy ring around the edge or tight button in the bottom of the cup The titre of a serum was read as the reciprocal of the highest dilution giving an 1+ reaction Titres ≧ 5 were considered positive

2) *Complement fixation test Cajdusek s modification* (*Gajdusek* 1958) of *Donnelly* s semi micro method (*Donnelly* 1951) was used with the modifications described in previous papers (*Vorup* 1965 1967) The *tissue antigens* used were sus

sions of normal human biopsy liver normal liver from rhesus monkeys and rotoxic th roid glands obtained at operation Two MHD of complement were used · titre of a serum was read as the reciprocal of the highest serum dilution giving more than 50 per cent haemolysis Titres ≧ 8 were considered positive

TABLE 3

Incidence of Rheumatoid Factors and Antibody to Thyroglobulin Distributed According to Sex in the Sera from Patients with Chronic Hepatitis and Normal Blood Donors

		Patients with Chronic Hepatitis			Normal Blood Donors		
		No of Sera			No of Sera		
	Sex	Tested	Positive	Percentage	Tested	Positive	Percentage
ex Fixation Test	♂	34	14	39 3	123	6	5 8
ositive Titre ≧ 20)	♀	73	28		66	5	
isitized Sheep Cell Test (Aler Rose)	♂	27	6	14 9	75	1	2 3
ositive Titre ≧ 40)	♀	67	8		53	2	
eptococcal Agglutination Test	♂	40	5	7 9	124	3	2 6
ositive Titre ≧ 20)	♀	86	5		68	2	
tibody to Thyroglobulin	♂	39	1	8 7	94	5	6 1
ositive Titre ≧ 5)	♀	88	10		53	4	

RESULTS

ible 3 shows the results of the serological tests for rheumatoid factor id antibody to thyroglobulin distributed according to sex from the itients with chronic hepatitis and healthy individuals As stated in this ble rheumatoid factors were demonstrated from three to seven times ore frequently in the patients than in the controls the latex fixation st and the S S C test reacting positively in 39 3 per cent (42 out of 07) and 14 9 per cent (14 out of 94) of the pathological sera and in 8 per cent (11 out of 189) and 2 3 per cent (three out of 128) respec ively of the normal sera The difference between the positive results patients and in donors is statistically significant both in the case of e latex fixation test (χ = 51 95 F=1 P < 0 1 per cent) and the S S C st (χ = 12 07 F=1 P < 0 1 per cent) [1] A positive SAT occurred in 9 per cent (10 out of 126) of the cases with chronic hepatitis and in 6 per cent (5 out of 192) of the donors (P < 5 per cent) Neither in e patients nor in the donors were rheumatoid factors correlated with x (the higher frequency of positive S S C tests and SAT in males with ronic hepatitis was not statistically significant)

[1] I am indebted to cand polit *S Ol sen I n B o* tatistical Department Statens eruminstitut f making the st tistical a l ses

TABLE 4

Results of Complement Fixation with Normal Human Biopsy Liver and Normal Liver of Rhesus Monkeys as Antigen Correlated with Rheumatoid Factors and Thyroglobulin Antibody in Sera from Patients with Chronic Hepatitis and Normal Blood Bank Donors

Liver Antibody Titres	Positive Reactions															
	Latex Fixation Test				Sensitized Sheep Cell Test				Streptococcal Agglutination Test				Tanned Red Cell Test			
	Patients		Normals		Patients		Normals		Patients		Normals		Patients		Normals	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
≤ 4	26/74	35	10/184	5	10/63	16	2/193	2	10/87	11	5/187	3	8/89	9	8/143	6
8–32	8/19	42	1/5	–	1/17	6	1/5	–	0/23	0	0/5	–	2/22	9	1/4	–
64–256	2/6	57			1/6	21			0/7	–			1/7	–		
≥ 512	6/8				2/8				0/9	–			0/9	–		
	43/107	39	11/189	6	14/94	15	3/128	2	10/196	8	5/192	3	11/127	9	9/147	6

Positive ≥ 8

TABLE 5

Incidence of Rheumatoid Factors and Antibody to Thyroglobulin Distributed According to the Aetiologic Type of Cirrhosis

Type of Chronic Hepatitis	Total Tested	Latex Fixation Test				Sensitized Sheep Cell Test			Streptococcal Agglutination Test			Tanned Red Cell Test		
		No of *Positive Titres*				No of *Positive Titres*			No of *Positive Titres*			No of *Positive Titres*		
		20	40 80	≧160	%	40 80	≧160	%	20 80	≧160	%	5–25	≧250	%
Posthepatitic	36	7	3	4	39	5	2	19	1	4	14	4	[illegible]	17
Cryptogenic	37	8	5	2	41	4	1	14		2	5	2	1	8
Alcoholic	15	4	1	1	40	2		13	1	1	13	1		7

Of the total of 6 cases with primary (one case) and secondary biliary cirrhosis two had a positive latex fixation test (both were cases of secondary biliary cirrhosis) none of the 6 cases reacted positively in the SSC test SAT nor the TRC test

Thyroglobulin antibody occurred with almost equal frequency in the sera from patients (8 7 per cent) as from donors (6 1 per cent) In the patient group a non significant higher frequency of positive TRC tests was found in the females whilst in the donors a positive TRC test occurred almost equally frequently in either sex

Table 4 shows the incidence of rheumatoid factor and antibody to thyroglobulin correlated with the incidence of complement fixing liver antibody in the sera from patients with chronic hepatitis and normal donors A positive latex fixation test occurred in 49 per cent (16 out of 33) of the sera reacting positively and in 35 per cent (26 out of 74) of those reacting negatively to liver antigen Rheumatoid factor was demonstrable by the latex fixation test in 8 out of 19 sera (42 per cent) having liver antibody titres from 8–32 and in 8 out of 14 sera (57 per cent) with titres $\geqq$ 64 Rheumatoid factor as indicated by a positive S S C test was found in 4 of 31 sera (13 per cent) with positive liver antibody titres and in 10 of 63 (16 per cent) sera with negative titres None of 39 sera which fixed complement in the presence of liver antigen and 10 of 87 (11 per cent) sera which failed to do so had a positive SAT ($P = 2\ 5$) a result which agrees well with the findings in the control group (none of five sera with positive liver antibody titres had a positive SAT) No relationship was found between the incidence of thyroglobulin antibody and complement fixing liver antibody

The incidence of rheumatoid factors and thyroglobulin antibody distributed according to type of chronic hepatitis is shown in Table 5 In all types of chronic hepatitis rheumatoid factor was demonstrated more often by the latex fixation test than by the S S C test and SAT No difference between the incidence of rheumatoid factors in the post hepatitic and cryptogenic group on the one hand and in the alcoholic group on the other was observed There was no correlation between high titres of rheumatoid factor and type of chronic hepatitis Thyroglobulin antibody occurred in 17 per cent (6 of 36) of the cases with posthepatitic cirrhosis and in 7–8 per cent of those with cryptogenic and alcoholic cirrhosis In most of the cases the titres were low (5–25) only one case of cryptogenic cirrhosis and myxoedema had a titre of 2500 and had also organ specific complement fixing thyroid antibody (titre = 32)

Table 6 shows the incidence of tissue antibodies and rheumatoid factors distributed by age in 94 patients with chronic hepatitis and 129 normal donors It can be seen that with the exception of the S S C test and SAT the incidence of positive tests had one maximum in patients aged 41 50 years and another one in those aged 61–70 years

In comparing the results of the serological reactions carried out on the patients and the donors the different age distribution of the individuals in the two materials must be taken into consideration since tissue antibodies and rheumatoid factors have been found more often in elderly than in younger individuals The difference between the frequencies of positive tests in patients and in donors is however too

TABLE 6

Positive Serological Reactions Distributed on Age of 94 Patients with Chronic Hepatitis and 122 Normal Blood Bank Donors §

Age	No. of Subjects Tested		Positive Cases: Complement fixing Liver Antibody		Complement fixing Thyroid Antibody		Thyroglobulin Antibody		Latex Fixation Test		Sensitized Sheep Cell Test		Streptococcal Agglutination Test		At Least One of the Antibodies Present	
	pat	don	pats	dons	pats %	dons	pats %	dons %	pats %	dons %	pats %	dons %	pats %	dons %	pats %	dons %
≤ 40	4	7[illegible]	(5)	[illegible]7	(0)	0.0	(0)	9.3	(50)	5.3	(25)	1.3	(0)	4.0	(50)	[illegible]0.0
41–50	1[illegible]	[illegible]	50.0	3.8	5.0	15.4	16.6	0.0	41.7	11.5	16.6	0.0	0.0	0.0	75.0	19.2
51–60	2[illegible]	17	[illegible]7	5.9	13.6	5.9	4.5	5.[illegible]	[illegible]7.3	11.8	18.2	5.9	9.1	5.9	50.0	[illegible]9.4
61–70	34	4	38.[illegible]	([illegible]5)	0.6	(0)	14.7	([illegible]5)	41.[illegible]	(25)	11.8	([illegible]5)	14.7	(0)	76.5	(25)
≥ 71	2		27.3		13.6		9.1		45.5		13.6		[illegible].1		[illegible]3.6	
	94	12[illegible]	33.0	4.1	17.0	4.1	10.[illegible]	7.4	39.4	8.[illegible]	14.9	[illegible].5	9.6	3.[illegible]	66.0	21.[illegible]

27 males and 67 females § 72 males and 50 females

great to be accounted for by this shift in the age distribution For example the incidence of complement fixing liver and thyroid antibody was significantly higher in the patients than in the donors (P < 0 1 per cent) In addition a non negligible percentage of positive reactors in the donor group was under 40 years a fact which may reduce the possible error resulted from the different age distribution of the two materials 66 0 per cent (62 of 94) of the sera from patients with chronic hepatitis

TABLE 7

Relationship between the Occurrence of Complement fixing Liver and Thyroid Antibody and Rheumatoid Factors as Determined by Various Techniques in the Sera from 32 Patients with Chronic Hepatitis

			Complement Fixation		*Rheumatoid Factors*		
Serial No	Age (yr)	Sex	Normal Human Biopsy Liver	Thyrotoxic Thyroid Glands	Latex Fixation Test	Sensitized Sheep Cell Test	Streptococcal Agglutination Test
1	64	♀	4096	2048	80	40	
2	69	♀	4096	2048			
3	65	♀	2048	2048	20		
4	60	♀	512	128			
5	57	♀	256	128			
6	50	♀	128	8			
7	49	♀	64	32			
8	70	♂	64	16			
9	85	♀	64	16	20		
10	72	♀	32	16			
11	49	♀	32	16	20		
12	58	♀	1024		20		
13	37	♀	1024		160	160	
14	62	♂	} 512		20		
15	55	♀					
16	44	♀	128		320	40	
17	75	♀	32		20		
18	76	♀					
19	64	♂	} 32				
20	67	♂					
21	67	♂	8		40	40	
22	80	♀		8	20	40	
23	46	♂			40	40	
24	54	♀			20	40	
25	69	♀	}		20		80
26	69	♀					
27	67	♂			1280	640	160
28	56	♂			640	320	160
29	76	♂			160	80	160
30	59	♂			160	40	80
31	66	♀			80	80	160
32	76	♀			20	40	160

All sera were tested for thyroglobulin antibody using the tanned red cell test Only one (case no 7) serum reacted positively (titre 1 25)

and 21 3 per cent (26 of 122) of the controls had at least one of the antibodies shown in Table 6 The difference in positivity between patients and donors was significant (P < 0 1 per cent)

Table 7 shows the incidence of complement fixing tissue antibody correlated with rheumatoid factor in the sera from 32 patients with chronic hepatitis It will appear from the table that sera which fixed complement in the presence of liver and thyroid tissue rarely had rheumatoid factor whilst on the other hand sera in which rheumatoid factor was demonstrable by at least two of the three tests rarely had complement fixing tissue antibody Of the 20 cases (Nos 1–20) which had complement fixing liver antibody at titres ≧ 32 three (Nos 1 13 16) reacted positively both in the latex fixation test and the S S C test All three cases were females two of them had posthepatitic cirrhosis (Nos 13 16) one had cryptogenic cirrhosis (No 1) Low titres (=20) of rheumatoid factor was demonstrated by the latex fixation test in 7 patients (one male and six females) Of these 4 (Nos 3 12 14 15) had complement fixing liver antibody at titres ≧ 512 and three (Nos 9 11 17) had titres of 32–64 Of the 6 sera (Nos 21–26) in which rheumatoid factor was demonstrable by two of the three tests one (a male with cryptogenic cirrhosis) had complement fixing liver antibody and one (a female with posthepatitic cirrhosis) had complement fixing thyroid antibody in both cases the titre was 8 In the sera from 6 patients (Nos 27–32) rheumatoid factor was demonstrable by all three tests In none of the six sera complement fixing antibody to liver and thyroid tissue were present It is noteworthy that some of the most hightitered sera of the whole material could be found in this patient group and that 4 of the 6 patients were males Three (Nos 27 29 31) of the six cases had posthepatitic cirrhosis one (No 28) had cryptogenic cirrhosis and two (Nos 30 32) had alcoholic cirrhosis

DISCUSSION

The presence of circulating tissue antibody associated with a marked hyper gammaglobulinaemia which can be seen in certain forms of chronic hepatitis has been assumed by some investigators (*Havens* 1959 1963 *Polish & Muschel* 1962) to be evidence of an enhanced activity of the reticulo-endothelial system In accordance with this assumption patients with chronic hepatitis have been found to respond more vigorous to a booster injection of purified diphtheria toxoid than patients without chronic hepatitis (*Havens et al* 1951)

The frequent occurrence of rheumatoid factors in chronic hepatitis which has been reported by earlier investigators may lend further support to the above mentioned concept (*Dresner & Trombly* 1959 *Howell et al* 1960 *Atwater & Jacox* 1963 *Caplan* 1963 *Butler & Paton* 1964 *Bouchier et al* 1964)

The results of the present investigation have confirmed that rheuma

toid factors occur with higher frequency in chronic hepatitis than in healthy individuals and that the latex fixation test reacts positively more often than the S S C test and the SAT In the present study no difference between the incidence of positive latex fixation tests and S S C tests in the cases of alcoholic cirrhosis on the one hand and in those of posthepatitic and cryptogenic cirrhosis on the other was observed

In this connection it is of interest that complement fixing liver antibody occurred almost exclusively in the cases of posthepatitic and cryptogenic cirrhosis (*Norup* 1967) a result which may suggest that different immunological mechanisms take part in the formation of rheumatoid factors and complement fixing liver antibody in chronic hepatitis

Only few investigations using the streptococcal agglutination test in chronic hepatitis have been undertaken In a larger material of hospitalized patients with various medical diseases excluding rheumatoid arthritis *Kalbak* (1948) found a positive SAT in 7 per cent (36 of 500) compared with 1 5 per cent of the sera from 64 healthy blood donors Similarly in the present investigation 7 9 per cent (10 of 126) of the sera from patients with hepatic cirrhosis and 2 6 per cent (5 of 192) of the donors had a positive SAT

The occurrence of thyroglobulin antibody in patients with chronic hepatitis has been assessed variously in different laboratories obviously owing to differences in the technique and antigens used Thus *Mackay & Wood* (1962) using a thyroid antigen for coating the tanned cells have observed positive TRC tests in 19 per cent (10 of 52) of cases of chronic hepatitis whilst *Skanse & Nilsson* (1961) who used purified thyroglobulin as antigen found a positive TRC test in 3 per cent (1 of 33) of sera from patients with chronic hepatitis In the present investigation thyroglobulin antibody was found with almost equal frequency in patients (8 7 per cent) as in donors (6 1 per cent) In this connection it should be mentioned that of 100 sera from patients with rheumatoid arthritis only 4 had antibody to thyroglobulin (*Norup* unpublished data)

Several investigations have been carried out with regard to the relationship between the incidence of tissue antibodies and sex and age It seems apparant from most of these investigations that non-organ specific tissue antibody are correlated to sex occurring more frequently in females than in males but not to age (*Hackett et al* 1960b *Kalas et al* 1963 *Serafini et al* 1964) and that organ specific tissue antibodies are correlated to both sex and age having been found more frequently in elderly women than in elderly men (*Goudie et al* 1959 *Hackett et al* 1960a *Hill* 1961 *Serafini et al* 1964) In the present series of patients complement fixing thyroid antibody occurred significantly ($P < 5$ per cent) more frequently in females than in males and a non significant increase of positive TRC tests in females aged 61–70 years was observed

Where the relationship of rheumatoid factor to sex and age is concerned the results are not consistent although studies carried out on large materials of sera from healthy individuals have suggested an increasing incidence of rheumatoid factor with increasing age in either sex (*Ball & Lawrence* 1961 *Serafini et al* 1964)

The poor correlation between the occurrence of rheumatoid factor and complement fixing tissue antibody having been found in the present series of patients is at present difficult to interpret In this connection it is of interest that a co existence of rheumatoid factor and complement fixing tissue antibody in patients with disseminated lupus erythematosus was not encountered more frequently than might be expected by chance thus of 37 patients 11 (30 per cent) had complement fixing tissue antibody 9 (24 per cent) had rheumatoid factor as demonstrated by the latex fixation test and only 7 (19 per cent) had both complement fixing tissue antibody and rheumatoid factor (*Norup* unpublished data)

The findings here presented seem to indicate that patients with chronic hepatitis produce gammaglobulins with rheumatoid factor activity in their reticulo endothelial system in amounts exceeding those which can occasionally be found in apparently healthy individuals Although the causes of this enhanced production of rheumatoid factor like globulins and moreover of antibodies to constituents of various tissues are not known their presence in serum seems to suggest that the primary lesion causing chronic hepatitis may be sought in the reticulo endothelial system of the patient

SUMMARY

Sera from patients with various types of chronic hepatitis and from healthy individuals were examined for rheumatoid factors using the latex fixation test the sensitized sheep cell (S S C) test and the streptococcal agglutination test (SAT) and for thyroglobulin antibody using the tanned red cell (TRC) test

A positive latex fixation test was found in 39 3 per cent (42 of 107) and 5 8 per cent (11 of 189) a positive S S C test in 14 9 per cent (14 of 94) and 2 3 per cent (3 of 128) and a positive SAT in 7 9 per cent (10 of 126) and 2 6 per cent (5 of 192) of the sera from patients with chronic hepatitis and from healthy blood donors respectively

No correlation was found between the incidence of rheumatoid factors and aetiological type of hepatic cirrhosis

By correlating the occurrence of rheumatoid factor and complement fixing tissue antibody a positive latex fixation test occurred in 49 per cent (16 of 33) of the sera reacting positively and in 35 per cent (26 of 74) of those reacting negatively to liver antigen a positive S S C test in 13 per cent (4 of 31) of the liver positive sera and in 16 per cent (10 of 63) of the liver negative sera Neither in patients nor in donors did

a positive SAT occur together with complement fixing liver or thyroid antibody

The incidence of thyroglobulin antibody in patients with chronic hepatitis did not differ from that found in the donors

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SERUM Pi TYPES IN NORWEGIANS

By

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Received 9 xii 66

The term Pi proteins was given to a system of serum proteins migrating faster than albumin during electrophoresis in acidic starch gels (4 5 6) The Pi proteins were found in five different phenotypes fitting a genetic theory of three codominant alleles called Pi^F, Pi^M and Pi^S

It has recently been shown (6) that the Pi proteins correspond to α_1 antitrypsin In ordinary starch gel electrophoresis α_1 antitrypsin migrates with the albumin When the pH in the gel is lowered to about 5 a series of protein zones—the Pi proteins—carrying the α_1 antitrypsin antigenic determinant appear in front of the albumin

The present report deals with the Pi phenotypes and Pi gene frequencies in Norwegians and the discovery of two new alleles

MATERIALS AND METHODS

Human sera Sera were obtained from 1043 healthy blood donors from the Red Cross Transfusion Centre Oslo and from 780 healthy blood donors and 1007 pregnant women from The Regional Transfusion Centre Stavanger Norway In addition blood samples were collected from 24 members of four families where new pheno types were observed Only one serum sample from each person in the Stavanger material was tested Sera giving other Pi band patterns than MM were re tested at least once In the Oslo material these persons were also re typed after new bleedings The 390 blood donors typed by *Fagerhol & Bræn l* (4) are not included in this ma terial The sera were stored below —20 C until tested

The donors at the Red Cross Transfusion Centre Oslo are predominantly citizens of Oslo but some are living in the suburbs Likewise most of the blood donors in Stavanger live in that city The transfusion centre in Stavanger receives blood samples for antenatal Rh blood typing and antibody screening from pregnant women living in south western Norway mostly from Stavanger and the county of Rogaland Since it has been shown (3) that the genes determining the α_1 antitrypsin types are not sex linked the blood donors and the pregnant women from Stavanger and Rogaland are treated together and referred to as the Stavanger population

Starch gel electrophoresis A modification of *Poulik*s (8) horizontal discontinu ous system was used with pH 4 95 in the gels The Pi typing is based upon the migration rates and the appearance especially the staining intensity of the Pi pro tein zones in front of the albumin

Conventional statistical methods were used The gene frequencies were found by direct counting of genes

The author thanks dr cent *Mikael Bræn l* The Veterinary College of Norway Oslo and dr *O Hartmann* for their stimulating interest in this work and for helpful criticism of the manuscript

RESULTS

A total of twelve different Pi phenotypes were observed A schematic drawing of them is presented in Fig 1 In the drawing the band patterns are stretched out to illustrate the relative positions of the protein zones in the different phenotypes The relative staining intensities are indicated by hatching increasing from white through hatched cross hatched and heavy hatching to black

The phenotypes FM MM, MS FS and SS have been described earlier (4) By technical improvement it has been possible to separate completely the F zones from the M zones in the FM phenotype as can be seen in Figs 1 and 3 The present study disclosed five new phenotypes which will be designated FF IM IS SZ and MV respectively

The phenotype called FF found in one person consists of one minor and two major protein zones of about the same staining intensities as the corresponding MM zones But the FF zones have higher migration rates and the distances between the zones are slightly shorter than between the MM zones In Fig 2 is shown that the parents and the two children of the FF propositus all have the FM phenotype It is therefore concluded that this propositus is homozygous $Pi^F Pi^F$

In the phenotypes IM and IS protein zones corresponding to the MM and SS zones are found respectively but with about half their staining intensities The IM and IS band patterns have three zones in common

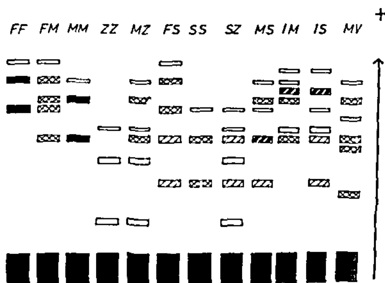


Fig 1

Schematic drawing of the twelve Pi phenotypes in front of the albumin Staining intensities are indicated by different shadings of black increasing from white through hatched cross hatched heavily hatched to black

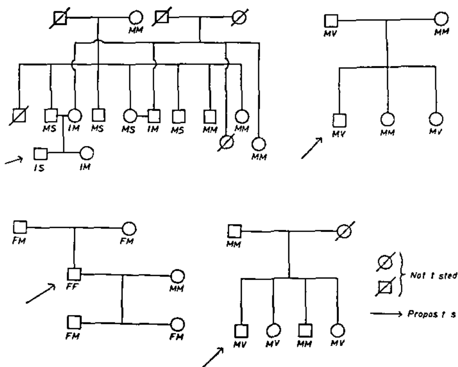


Fig 2
Pi phenotypes observed in four families where new phenotypes were found

which are specific for these phenotypes one very weak zone in front one relatively strong zone (zone no 2 in the IS phenotype) and one weak zone closely in front of the slowest MM zone The relative positions of these three zones correspond to the MM band pattern except that their migration rates are intermediate between those of the FF and MM protein zones In Fig 2 is shown the Pi phenotypes observed in the family of the IS propositus Protein zones corresponding to the intermediate band pattern were found in three of his relatives It is therefore concluded that the band patterns IM and IS are heterozygous phenotypes between a new allele for which the term Pi^{I} is proposed and the alleles Pi^{M} and Pi^{S} respectively The general pattern of the allele products is one weak zone in front of two major zones of about equal staining intensity It is evident that Pi^{I} allele product deviates from this general pattern since the slow major zone is much weaker than the major zone in front

The phenotype called MV consists of two weak and four major zones with about half the staining intensity of the MM zones The two major zones in front correspond to the MM zones while the two major zones behind have lower migration rates than the SS zones The distance between the two slowest MV zones is slightly longer than between the

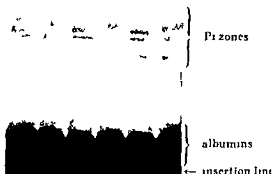


Fig. 3

Photograph of an amido black stained starch gel showing the Pi phenotypes IM and FM

major SS zones. In Fig. 2 is given the Pi phenotypes found in two unrelated families where the phenotype MX was observed. It is concluded that the MX phenotype is a new heterozygous type resulting from the combination of the allele Pi^M and a new allele for which the term Pi^X is proposed.

It has been shown (6) that sera from persons who are homozygous for α_1 antitrypsin deficiency give Pi band patterns with slower migration rate than the M band pattern. In addition the protein zones are very weak corresponding to about 10 per cent of the normal α_1 antitrypsin concentration in serum. The term Pi^Z was therefore chosen for the allele for α_1 antitrypsin deficiency (6). Only when at least 5 times the normal amount of serum is applied in the starch gel for Pi typing can the ZZ zones be seen. Even then the protein zones are weak. The sera which on repeated testing (using normal amounts of serum) gave no clearly visible protein zones in the Pi region were classified as phenotype ZZ. Those sera which on repeated testing gave MM or SS zones with about half the normal staining intensities as judged by the unaided eye were classified as MZ and SZ respectively.

Fig. 3 is a photograph of an amido black stained starch gel where the phenotypes FM and IM are compared and in Fig. 4 is shown the new phenotype MX compared with MM, MS and SS. On this photograph the weak zone belonging to the X band pattern can not be seen as it disappears between the M zones. The optimal conditions for separation of the partially overlapping phenotypes FM and IM are somewhat dif-

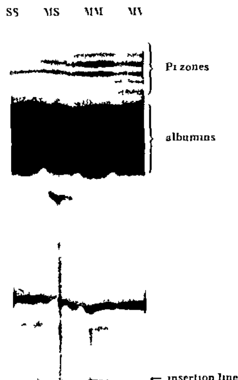


Fig 4

Photograph of an amido black stained starch gel where the phenotypes SS MS MM and MV are compared

ferent from the optimal conditions for slow heterozygous types as MV The gels shown on these two photographs were therefore run under somewhat different conditions as can be seen from the positions of the albumins

The protein zones resulting from Pi^S are much weaker than those resulting from the other alleles except Pi^Z This observation has been made on all SS MS IS and FS phenotypes studied Quantitative data are not yet available but studies in this respect are in progress

In Table 1 is given the distribution of Pi phenotypes in 1781 persons living in the Stavanger area and 1043 persons living in the Oslo area and in the total of 2830 Norwegians As can be seen the observed and expected number of individuals of the different phenotypes are quite similar In Table 2 is given the gene frequencies found in the same populations It is evident that the gene frequencies found in Stavanger population do not differ significantly from those in the Oslo population These two populations were therefore added and the gene frequencies calculated for the total material of 2830 Norwegians

TABLE

Observed and Expected Pi Phenotypes in 1787 Persons Living in the Stavanger Area,

		MM	MS	FM	MZ	IM	FF	MX
Stavanger	Observed	1600	80	47	44	5	1	1
	Expected	1595	85 1	46 27	48 97	4 778	0 3354	1 013
Oslo	Observed	940	36	25	37	1	0	1
	Expected	939 6	38 01	24 75	35 04	1 98	0 163	0 99
Total	Observed	2540	116	72	81	6	1	2
	Expected	2534	123 2	71 24	84 09	6 427	0 5006	2 [illegible]

TABLE 2

Gene Frequencies Observed in the Stavanger and Oslo Populations and in a total of 2830 Norwegians

	Pi^M	Pi^S	Pi^Z	Pi^F	Pi^I	Pi^X
Stavanger	0 9449	0 0257	0 0145	0 0137	0 0014	0 0003
Oslo	0 9491	0 0192	0 0177	0 0125	0 0010	0 0005
Total	0 9463	0 0230	0 0157	0 0133	0 0012	0 0004

DISCUSSION

It is seen from Table 2 that the Pi system consists of one common allele Pi^M and several more or less rare alleles According to the genetic theory 21 different phenotypes are expected for six alleles but only twelve have been found yet The allele Pi^X described by *Axelsson & Laurell* (1965) and retested by *Fagerhol & Laurell* (1967) were not found in this study From the gene frequencies it is seen that the phenotypes FI II FX IZ IX SX XX and XZ must be very rare in Norwegians and none of these were observed The phenotype FZ was not found but this is probably a matter of chance since the expected number was only 1 1182

Axelsson & Laurell (1965) have presented evidence including family studies that Pi^Z and Pi^X are alleles to Pi^M The distribution of phenotypes as well as the family studies presented in this report support the genetic theory advanced (4) that the Pi system consists of several codominant alleles Exceptions to this mode of inheritance have not been observed

Several authors (2 7 9) have found that α_1 antitrypsin is unstable to heat and low pH The observation that the Pi zones carrying the antigenic determinant of α_1 antitrypsin appear in front of albumin as the pH is lowered towards 5 in starch gel electrophoresis suggests that the Pi zones represent split products of α_1 antitrypsin If this is true

1

in 1043 Persons Living in the Oslo Area and in the Total of 2830 Norwegians

SZ	SS	FS	IS	ZZ	FZ	χ	n	P
4 1 306	3 1 135	0 1 234	0 0 1?61	2 0 3757	0 0 71	3 15	4	0 6>P>0 5
0 0 7089	1 0 384o	1 0 5006	1 0 0401	0 0 3?68	0 0 4615	0 27	4	0 99o>P>0 99
4 2 044	4 1 497	1 1 731	1 0 156?	2 0 6976	0 1 182	3 15	5	0 7>P>0 6

the protein corresponding to Pi^I must be split into two major polypep tides of unequal molecular weights while splitting of the other allele products gives major polypeptides of about the same size The chemical basis of the different Pi phenotypes is not known but might well de pend upon substitution of one or more amino acids The difference in staining intensity of the major zones resulting from Pi^I suggests that there may be more than one point of substitution in the protein molecule

Eriksson (3) has shown that there is a close relationship between the phenotype ZZ (homozygous state of α_1 antitrypsin deficiency) and chronic obstructive broncho pulmonary disease Whether other Pi phenotypes may predispose for disease remains to be studied

SUMMARY

Serum Pi types were studied in 2830 Norwegians by starch gel electro phoresis Twelve different phenotypes were observed fitting a genetic theory of six codominant alleles called Pi^F Pi^I Pi^M Pi^S Pi^V and Pi^Z Band patterns corresponding to the new alleles Pi^I and Pi^V are de scribed The symbols for the allele products were chosen according to the migration rates of the proteins which decrease in the following order F I M S V and Z In this material the following gene frequen cies were found Pi^M 0 9463 Pi^S 0 023 Pi^Z 0 01o7 Pi^F 0 0133 Pi^I 0 0012 Pi^V 0 0004 The allele products of Pi^F Pi^M Pi^S and Pi^V appear as a three band pattern one weak zone in front of two major zones The alleles Pi^Z and Pi^I give exceptional patterns Pi^Z gives only about 10 per cent of the normal protein concentration while Pi^I gives major zones of unequal staining intensities

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EXPERIMENTAL STUDIES ON BOVINE TICK BORNE FEVER

1 *Clinical and Haematological Data some Properties of the Causative Agent and Homologous Immunity*

By

JAAKKO TUOMI

Received 20 xii 66

Tick borne fever (reviewed by *Tuomi* (17)) was first described as a disease of sheep later the condition was also detected in cattle In the main earlier studies of both ovine and bovine tick borne fever were made in Great Britain The occurrence of bovine disease has additionally been reported from Ireland Norway the Netherlands and Finland To date the only major experimental study of bovine tick borne fever published has been the original paper by *Hudson* (11)

In Finland experimental and field studies of bovine tick borne fever have been undertaken since summer 1964 The findings in epidemiological field studies have already been reported (17) as have those of an electron microscopic examination of the structure of the causative agent which led to the suggestion that this agent occupied a taxonomic position in the middle ground between typical rickettsiae and organisms of the psittacosis group (20) The present series of papers contains reports on most of the experimental findings made so far The first paper describes clinical features in general the occurrence of tick borne fever bodies haematological data carrier state immunity to homologous strain preservation of the agent and studies concerned with its cultivability and host range The subjects of the following papers are strain differences in virulence strain differences in immunological properties the related character of strains as revealed by immuno fluorescence technique and the susceptibility of the agent to antibiotics and sulphonamides

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This work w[illegible] rted by g[illegible]t from *the Finnish Medical Research Council* and from the *M[illegible]try of Agri ult[illegible]* Grat ful ackno ledg ment is due for the opportunity t[illegible] u[illegible]ttle f the *D[illegible]t of Obstetrics and Gynaecology* (College of Veterinary Med[illegible]n) in Hautj[illegible] n ily provided by the Head of th Department Profe or *R M[illegible]g* and [illegible] ta e in the determination of haematological data by Mrs *E[illegible] Marja H[illegible]r*

smears from the yolk sacs and occasionally from other membranes as well from embryo tissues and from blood cells of developing and hatched chickens From five series of eggs each at a different level of blind passage yolk sac suspensions in sucrose PG (1) were inoculated into susceptible cattle

Primary calf kidney cells maintained in a medium (Eagle s minimum essential medium +2 5 per cent calf serum +20 per cent tryptose phosphate broth) devoid of antibiotics were once inoculated with blood infected by J34 E4 strain At 14 days a mixture of cells and medium was inoculated intravenously into a susceptible cow HeLa and BS C cells were inoculated once and a blind passage was carried out

RESULTS

Clinical Features

The following description of clinical features is based upon reactions occurring in animals with no previous experience of tick borne fever No more than partial reference is made to strain differences as regards the virulence

Table 1 lists some data in respect of 36 cattle and of their reactions to the tick borne fever agent

In 35 cattle the incubation time varied from 4 to 11 days with a mean of 6 9 days As is observable from Table 1 several inocula had been stored in different ways before injection this procedure often obviously prolonged the incubation time In cow 22 on which nymph of *I ricinus transmitting the J Puhe strain* had been allowed to engorge the incubation period was 12 days The most conspicuous clinical sign was fever The recorded maximum temperatures in 36 cattle ranged from 39 7 C to 41 8 C (mean 41 0 C) The form of the fever curve was mostly rather even with a rapid rise to the maximum and a gradual diminution however at times great *fluctuation was evident (Fig 2) The duration of fever ranged from* to 5 days (mean 3 5 days) Secondary temperature rises lasting from one to two days appearing 3 to 8 days after subsidence of the primary reaction were recorded in 9 out of 36 animals

Concurrently with the fever cattle showed other signs of illness although as a rule these were disproportionately mild in comparison with the high fever Most animals appeared slightly to moderately depressed with diminished appetite but only during the height of the temperature reaction As a rule the respiratory rate increased but only exceptionally to more than twice the normal The pulse rate was only slightly elevated as a general rule All but two of 20 cattle kept under close observation at the State Veterinary Medical Institute were found to cough *during the illness Cows 21 and 22 which did not exhibit this symptom* reacted to the less virulent J Puhe strain (cf next paper of the series (18)) Several of those kept outside Helsinki were also reported to have coughed Coughing seemed to be delayed when taken against the fever reaction it started during its latter part or after it had subsided Coughing was mostly mild dry and infrequent it could frequently have easily escaped notice if not made the object of specific observation Only occasionally did coughing last longer than for two three days and then usually became louder as it progressed Simultaneously with coughing some animals had discharges from the eyes and nose Loosening of the consistency of the faeces was noticed in two animals Two adult cows stood with curved back and frequently lifted their hind feet

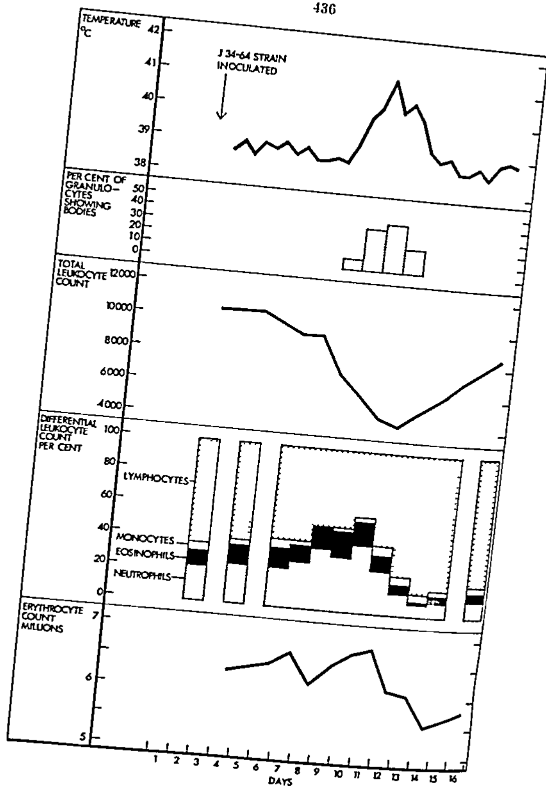
In all the eight cows lactating at the time of reaction there took place drop in the milk yield *ranging from slight to* less than half of the previous level In only one cow was the daily yield measured A fall from 17 l to 9 l was concurrent with the temperature reaction The former level was regained in 5 days In all cows at least nearly full milk production was restored within 2 weeks Not one of the pregnant cows or heifers aborted in connection with tick borne fever and all of them later had normal parturition in the due time

Table 1 indicates that adult cattle tended to be affected more markedly than calves although all the calves even that one week old exhibited a definite reaction

D ta of T k l rn F t Re ct on n 36 (a ll n th no Farl er Exper n e of the D se

Designation of the animal	Age	Blood Inoculated: Strain of agent	Way of storage	Length of storage	Incubation time days	Temperature reaction: Max temp C	Duration: Primary days	Secondary days	Occurrence of tick borne fever bodies: Primary days	Secondary days	Maximum percentage of granulocytes showing bodies
1	r	J34 64	+4 C§	2 d	5	40 5	3		4		22
C lf	n			3 d	5	40 9	4		4		6
Calf 3	3 m		74 C§	1 mo	6	40 8	4	2	6	4	30
Calf 4	4 m			1 mo	9	40 0	2		3		10
Calf 5	3 mo		+4 C	4 d	6	40 3	2		2	1	12
Calf 6	1 w	Ju3 64	§	3 d	7	41 0	3		4		42
Cow 7	old cow	J48 64	§	3 d	7	40 4	3	1	4	2	15
Cow 8	old cow	J34 64	74 C	3 mo	8	41 25	3		4	2	38
Calf 9	10 m			4½ mo	10	41 0	2		2		16
			+4 C	3 d	6	41 6	3	2	3	1	12
			74 C	1 mo	7	40 5	2		2		11
Cow 1			Fresh		11	40 7	5		4		32
~~Calf 13~~			−74 C	2¼ mo	7	41 15	5		6	1	39
~~Calf 14~~			Fresh		4	41 1	4		8		48
C	1		−74 C	3 w	7	41 7	3	1	4†	n e	32
Cow 16	5 y			1 ¼ mo	6	41 5	5		4		60
Cow 17	3½ y			3 w	7	41 8	3		4†		58
Heifer 18	2 y			3 w	7	40 7	3		4†		40
Cow 19	3 y	"		3 w	7	41 4	2		4†		36
Heifer 20	2 y			3 w	7	41 4	2		4†		48
Cow 21	3 y	J PuKe	Fresh		5	41 2	4		2		2
Cow 22	old cow		(Ticks)		12	39 7	4		2		4
Calf 23	9 mo	J1 65	+4 C§	3 d	9	41 2	3		3	2	18
Heifer 24	1½ y	Jo5 65		4 d	10	40 8			7		6
Calf 26	5 mo		Fresh		5	40 9	5		5		28
Calf 27	9 mo	J34 64	−74 C	1¼ mo	8	40 3	2		1		16
Cow 28	4 y	J PuKe		1 ¼ mo	7	40 6	4		2		10
Cow 29	3 y			1½ mo	10	40 65	4		1		2
Cow 30	3 y	K20 65		1½ mo	5	41 1	4		5		56
Cow 31	3 y			1½ mo	5	41 [illegible]	4	2	4†	n e	68
Cow 32	old cow	"		1 ¼ mo	6	40 65	5		4†		44
Heifer 33	1½ y			1 ¼ mo	6	41 3	5	1	4†	n e	52
Heifer 36	1½ y	J1 65		1 mo	4	41 7	4	1	7	1	20
Heifer 37	1½ y	K1 65		4 ½ mo	7	41 45	3	1	6	1	72
Heifer 38	1½ y	J34 64		5¾ mo	6	41 4	5	2	4	2	32
Calf 40	10 mo	T1 65	§	2¼ m	7	40 8	4		3		48

= splenectomized § = blood from the field case † = not further examined n e = not examined



TEMPERATURE °C
42
41
40
39
38
J 34-64 STRAIN INOCULATED
PER CENT OF GRANULOCYTES SHOWING BODIES
50
40
30
20
10
0
TOTAL LEUKOCYTE COUNT
12000
10000
8000
6000
4000
DIFFERENTIAL LEUKOCYTE COUNT PER CENT
100
80
60
40
20
0
LYMPHOCYTES
MONOCYTES
EOSINOPHILS
NEUTROPHILS
ERYTHROCYTE COUNT MILLIONS
7
6
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DAYS

Fig 4

end of the temperature reaction the number of lymphocytes again began to rise but the diminution in granulocytes continued At the point when examinations were ended a rise in the number of granulocytes had also commenced As regards the granulocytes the numbers of both neutrophiles and eosinophiles seemed to be af fected in the same way No effect on the basophil numbers was observable Im mature neutrophils and even eosinophils were often detected during the diminution phase of granulocyte numbers At one point in cow 17 the number of band neutro phils reached almost half the number of the mature ones A slight absolute but not relative rise in the number of monocytes is indicated as having occurred at the end of the clinical reaction and lasting for a few days A corresponding and some times more marked increase in the number of monocytes was encountered in several other cattle examined The other findings of these unpublished examinations re semble in principle those presented in Fig 4 It is further seen from the same Figure that the erythrocyte count fell slightly after the end of the clinical reaction

Carrier State

The subinoculation of fresh blood obtained during the period in which tick borne fever bodies were detected microscopically regularly resulted in tick borne fever reaction in susceptible animals With a view to determining the length of the period during which blood usu ally remained infective after the microscopical disappearance of bodies there were undertaken tests indicated in Fig 5

In all the animals except calf 2 which had also reacted to another strain the pre ceding reaction had been caused by J34–64 strain In every case but one 3 to 6 ml of fresh blood was inoculated intravenously however the positive reaction of the blood from heifer 13 obtained 9 days after the disappearance of bodies resulted from the inoculation of 500 ml of blood this was furthermore the only subinocula tion which brought about tick borne fever reaction All the other tests with blood taken between 3 days and 8½ months after the reaction were negative as were the two splenectomies

There was no specific intention of studying the carrier state after reactions caused by other agent strains Nevertheless on one occasion such a state was en countered accidentally The blood of sheep 2 (splenectomi ed animal) was found to carry J–Puhe strain after the elapse of approximately one month and one week from subsidence of the clinical reaction caused by this strain This finding will be described in greater detail in one of the later papers (19) Later papers will also show that J Puhe strain is highly virulent in sheep (18 19)

Immunity to Homologous Strain

An examination was made of the development and duration of homo logous immunity for 6 various strains in both cattle and sheep by the application of challenge tests The results obtained are presented in Fig 6

Most tests were concerned with immunity against J34–64 J-Puhe and K20–65 strains On none of the 30 occasions in which the first challenge was effected within 3 months (the majority were within 3 weeks) after subsidence of the preceding reac

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Fig 4

A composite chart temperature reactions occurrence of tick borne fever bodies and haematological data in three cattle (cows 17 18 and 20) inoculated simultane ously with J34 64 strain The values are means from the three animals

Preservation of Infectivity in Stored Blood

In a number of tests it proved that the infectivity of blood obtained at the height of a primary tick borne fever reaction seemed to be preserved regularly up to 7 days when the sample had been stored at +4 C. Blood infected with J Puke strain once caused tick borne fever after storage for 14 days and once after 1 month of storage. In the latter case the incubation time was extended to seven days. With fresh blood the incubation period of J Puke strain in sheep usually varied between 2 and 4 days (18). On one occasion blood obtained from a reaction caused by J Puke strain proved negative after storage for 3 weeks. In summer 1966 a new Kingasniemi strain was found to be infective after 14 days storage of the blood.

It was demonstrated that storage at —74 C regularly preserved the infectivity of blood obtained at the height of the reaction and frozen immediately for a period of several months at least. Table 2 lists the positive inoculations with blood stored in the frozen state.

These include samples each infected with one of seven different strains. The longest preservation recorded (J34-64 strain) was 8 months. An unnoticed fault in the deep freeze box which resulted in the thawing of samples precluded tests on preservation extending over 8 months. Inferior preservation in the frozen state was recorded when blood received from the field (6 cases) or blood stored for several days at +4 C was frozen. Only 2 blood samples from the field, one of which contained J34-64 strain and the other T1-65, were found to be infective after respectively 1 and 4 months storage at —74 C.

Both the storage of blood at +4 C and the freezing of blood and subsequent storage at —74 C tended to extend the incubation time beyond that recorded for fresh blood. Table 2 provides information in this regard on tests made with blood containing J34 64 strain.

TABLE 2

Length of Incubation Period in 19 Cattle and 5 Sheep Inoculated with J34-64 Strain and with no Previous Experience of Tick Borne Fever by Various Ways of Storage

Length of incub period days	Number of animals reacting to inoculation with						
	Fresh blood	Blood stored at +4 C, days		Blood stored at − 74 C, months			Total
		1–2	3–5	1–2	2–4	4 8	
4	2						2
5		1	1				[illegible]
6	1	1	1	2	1	1	7
7		1		7	2		10
8				1	1		2
9				1			1
10						1	1

Only inoculations with blood obtained at the height of the reaction are taken into consideration. = includes one sheep

Tests of Cultivability and of Host Range of the Agent

Negative results were recorded in all attempts made to propagate the agent in conventional bacteriological media, tissue culture, embryonated hens eggs, white mice, guinea pigs, pigs, a horse and an elk calf. No structures which simulated tick borne fever bodies were detected. Only the two pigs exhibited a slight temperature rise 8 days after the inoculation, even in their blood, however, no tick borne fever bodies were detected. Subinoculations of pig blood were not carried out. Subinoculation of the horse blood and the three subinoculations of the blood of the elk calf were all negative. No cytopathic effect was observable in the tissue cultures and the calf inoculated with cells and medium from the calf kidney culture displayed no reaction.

DISCUSSION

Some of the symptoms exhibited by affected cattle deserve special attention. One interesting finding was the regular association of mild coughing with the condition of tick borne fever in animals which were inoculated with strains isolated from bovine field cases and kept under sufficiently close observation. *Hudson* (11) and *Øveras & Hunshamar* (13) recorded coughing in some of the affected cattle but considered that this might be merely coincidental. The present results exclude coincidence as the explanation of this association and strongly suggest that coughing is part of the typical syndrome of bovine tick borne fever. It has been previously reported that coughing was recorded in 41.9 per cent of 310 field cases of bovine tick borne fever (17). A reminder is necessary here that if close attention is not paid to the cough, often silent, it may easily escape notice.

The pathogenesis of coughing in tick borne fever remains to be established. It does not seem probable that it is directly effected by the tick borne fever agent. British workers who made histological examinations of various organs of sheep and cattle killed during the disease attack found tick borne fever bodies in only blood granulocytes and monocytes (9, 11). *Foggie* (3, 4, 5) has proved that an attack of ovine tick borne fever greatly diminished the resistance of sheep to staphylococci; he considered that the impaired defence was attributable to the destruction of granulocytes by the tick borne fever agent. Coughing in bovine tick borne fever coincides with the later part of the clinical infection, at which stage the number of granulocytes is at its lowest. It could be thought that the shortage of granulocytes might provide an opportunity for the normally nonpathogenic bacteria which inhabit the respiratory tract to cause a mild pathological reaction. The development of discharges from the eyes and the nose might have a similar explanation.

The findings in the present study support the impression derived from the results obtained in the field study (17) that Finnish strains

of bovine tick borne fever agent might in general be less apt to cause abortion than the Scottish strain described by *Wilson et al* (21)

In tick borne fever one frequent phenomenon is the appearance of a spontaneous relapse In experimental cattle the interval between the end of the primary reaction and the appearance of the secondary reaction ranged from 3 to 8 days However in the field study material the time interval that is the period between the observed commencement of both reactions varied from 11 to 18 days (17) Despite the difference in the ways of recording in the field material the actual interval was on the average definitely longer Against this in three experimental sheep infected by J Puke strain intervals from 7 to 13 days were recorded a range which roughly corresponds to that in the bovine field material As was mentioned above J Puke strain is highly virulent in sheep and the relapses stated were more severe than those recorded in the experimental cattle Under field conditions the milder relapses of cattle might very well have gone unnoticed The above findings may suggest that there exists a correlation between the length of the interval and the severity of both primary and secondary reactions this may explain why only those relapses which appear after a relatively long interval are recorded under field conditions

An intriguing question to which the answer can at present to a great extent be no more than a matter for conjecture is what makes the primary reaction subside and why this inhibition is occasionally so short lived that a secondary reaction will emerge? Such phenomena can hardly be explained in terms of antibody mediated immunity It is more likely that the development of some kind of auto interference of limited duration is responsible for supression and interruption of the reaction The subject will be further discussed in the next paper of the series (18)

In principle the haematological findings correspond to those of *Taylor Holman & Gordon* (16) in connection with ovine tick borne fever

The successful subinoculation by 500 ml of blood obtained 9 days after the reaction and the simultaneous failure by 3 ml of blood emphasize that in all probability the conventional method of inoculating 3 to 6 ml was quite inadequate to reveal a low level carrier state and consequently no answer can be given to the question whether such a state was present in the investigated cattle Splenectomy has been described as activating a latent tick borne infection in sheep (2) Splenectomies of calf 4 and sheep 2 were negative in this respect and may suggest that in these animals prior sterilization had taken place

It seems apparent that strains of tick borne fever agent differ in their capability to induce a carrier state or at least in regard to the level of the state which they induce The following facts appear to support this contention In the present study J Puke strain which is highly virulent in sheep was recovered from the blood of sheep 2 about 1¼ months after subsidence of the clinical reaction (the inoculum was

1 ml) In ovine tick borne fever caused by British strains which is usually a relatively severe affection a carrier state at least several months in duration was a frequent finding when subinoculations were made with a few ml of blood (2) On the other hand the Indian rather mild variant of the ovine tick borne fever agent was never recovered from the blood once more than 4 days had passed since the end of the clinical reaction (14) This information may suggest the existence of correlation between the virulence of the strain and the duration or level of the induced carrier stage As regards bovine tick borne fever the only mention of examination of the duration of the carrier state is that made by Hudson who found that blood obtained from a bovine 54 days after inoculation was negative (11) It cannot be known how far J34 64 strain typifies the behaviour of other Finnish bovine strains in the ability to remain latent but it is likely that they are inferior to British ovine strains in this respect

To judge from various reports the immunity caused by Finnish strains appears to be more solid and durable than that induced by most other strains investigated in this regard The only bovine strain on which data are available is that isolated by *Hudson* (11) Hudson reported that 11 out of 14 animals re inoculated six to twelve months after previous experience of the disease reacted like fully susceptible cattle He also mentioned that under natural conditions the disease is said to attack cattle quite commonly in successive lactation In Finland where the winter period during which cattle are housed and ticks are inactive is much longer than in England recurrence of the disease in later grazing seasons is nevertheless rare (17)

That strains may differ in the degree of protection they induce is also suggested by the conflicting findings made when different strains of ovine agent have been studied (2 10 15) A finding and clearly at variance with the Finnish experience was that the resistance to re infection by an ovine agent strain did not develop earlier than about 5 weeks after the primary infection (2)

The discovery that the agent can be preserved in the frozen state offered better opportunities for further studies It would not have been possible to make the immunological comparisons of several strains reported in the third paper of the series (19) if the only way to preserve strains had been continuous passage in experimental animals Recently *Foggie Lumsden & McNeillage* have also reported the successful preservation of ovine tick borne fever agent in the frozen state (8) These authors however used dimethyl sulphoxide or glycerol as preservative

The negative results of the attempts to propagate tick borne fever agent in systems other than those of cattle or sheep are complementary to previous findings by other workers (2 5 11) Although *Fogie & Hood* (7) succeeded in adapting the ovine agent to multiply in splenectomized guinea pigs and mice and subsequently also in non splen

ectomized guinea pigs the virulence as regards these animals remained low A rather strict species specificity seems to be a characteristic of tick borne fever agent

By virtue of the related nature of the animal species concerned it was slightly surprising that the elk calf did not detectably react to any of the three strains inoculated In Great Britain tick borne fever infection has been demonstrated in both red deer (5) and in fallow and roe deer (12) However it cannot be concluded on the basis of this limited experiment that elk is not susceptible to tick borne fever Both the age (11) of the calf and the possible non virulence to elk of these specific strains might be sufficient to explain the negative result obtained here

SUMMARY

The reaction to tick borne fever agent in 36 experimental cattle was characterized by moderate to high fever slight to moderate depression and diminution of appetite All the cattle inoculated with bovine strains and kept under close observation exhibited a mild cough Some animals displayed further symptoms

In rough coincidence with the fever reaction tick borne fever bodies were demonstrated in blood granulocytes and occasionally in monocytes as well A short reappearance of bodies often associated with fever was observed in 10 out of 36 cattle

Granulocytopenia was the most conspicuous of the haematological findings The lowest counts were at about the end of the fever reaction

No carrier state was apparent in 6 cattle and 2 sheep each infected with J34 64 strain when subinoculations were made with 3 to 6 ml of blood obtained between 3 days and 8½ months after th end of the clinical reaction However an inoculum of 500 ml taken 9 days after the end of reaction caused tick borne fever Additionally on the subinoculation of 1 ml of blood a splenectomized sheep was found to carry J-Puke strain after 1¼ months

In none of the 30 animals did challenge by a homologous strain within 3 months induce a detectable reaction Two out of 5 animals which were challenged later on did react but only after a clearly prolonged incubation period

On one occasion blood stored at +4 C for one month resulted in infection Shell freezing of blood and subsequent storage at —74° C could preserve the infectivity for at least 8 months

Attempts to propagate the agent in systems other than cattle and sheep were unsuccessful

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SENSITIVITY OF VARIOUS SALMONELLA STRAINS TO FELIX 0–1 PHAGE

By

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Received 28 xii 66

The bacteriophage 0–1 of *Felix & Callow* (1943) has been found to act with a high degree of specificity on salmonella bacteria (*Cherry et al* 1954) When tested on more than 2 000 strains (*Thal & Kallings* 1955) 99.5 per cent of the salmonella cultures were lysed as opposed to only 0.3 per cent of the non salmonella members of the Enterobacteriaceae family Due to this specificity the 0 1 phage has been used as an additional tool for the routine identification of salmonella bacteria in Sweden The usefulness of the 0–1 test has been found to vary a great deal with the technique applied and the type of material tested The effective multiplicity of infection for various salmonella strains is described in the present paper The results are analyzed in connection with the ability or inability of the bacteria to support virus growth

MATERIALS AND METHODS

Bacteria The strains were isolated from clinical specimens or originated from cultures submitted to the National Bacteriological Laboratory for typing When not tested directly they were stored in sealed agar stab tubes at room temperature If not otherwise stated cultures grown in broth for four hours at 37° C were used for experiments The bacterial concentration was estimated by colony counts or in some cases by the aid of a nephelometer standardized by colony counting

Phage Felix 0–1 phage was propagated on strain *B76* *S paratyphi B* phage type 1 by the agar layer method Sterile lysates filtered through Jena sintered glass filters and stored undiluted at +4° C were used as stock suspensions The number of infectious phage particles was estimated by the standard agar layer method 1.0 ml phage dilution was pipetted into a tube containing 8.5 ml 0.6 per cent melted agar at 45° C to which 0.5 ml cultures of B76 had just been added After mixing 2 ml were poured over an agar plate containing 1.0 per cent agar medium The plaques were counted on the following day after incubation at 37° C overnight The absolute efficiency of plating was unknown but with regard to the high plaque counts (10^{11}) it was assumed that the plaque count corresponded closely to the true number of infectious particles

As a standard method for spot tests a loopful of bacterial broth culture was inoculated over a circular area (17 mm diameter) on 1.0 per cent agar plates by a platinum loop of 2 mm interior diameter giving an average drop volume of 0.005 ml Using the same kind of standardized loop the phage dilution was spotted on the inoculated areas after they had dried The phage drop covered a circular area of

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Preliminary report given 1.X.1961 to the Medical Microbiology Division of the Swedish Medical Society (*Kallings* 1962)

With the technical assistance of Mrs *Maud Lindberg* and Mr *Gerd Lundström*

about 9 mm diameter The readings were taken after five hours incubation at 37 C. The multiplicity of infection obtained with this technique is calculated in Table 1

The one step growth curve on the propagating strain of phage 0–1 had been determined by repeated experiments in order to learn some of the characteristics of the phage At a virus concentration of 0 2 phage particles per bacterium (3×10^8 viable cells per ml) on an average 40 per cent of the phage particles were adsorbed to the host cells within 10 minutees at 37 C Phages were first liberated 25 minutes after infection and the titer increased over a period of 10 minutes The burst size was about 220 infectious particles per bacterium.

Media The same Difco nutrient agar and broth as described in a previous paper (*Kallings* 1961) was used if not otherwise stated

Diluents Nutrient broth (Dif o) or phosphate buffered saline pH 7 4

TABLE 1

Calculated Multiplicity of Infection with the Standard Spot Test Technique

	Titer of suspension	Spot diam mm	Particles or cells per loop	Particles or cells per mm
Phage	$1\ 9 \times 10^{11}$	9	$1\ 7 \times 10^{3}$	$[illegible]\ 7 \times 10$
Bacterium	$1\ 0 \times 10^{8}$	17	$8\ 8 \times 10$	$3\ 9 \times 10^{3}$

Resulting phage to bacterium ratio $6\ 9 \times 10^{3}$
Figures taken from the experiments summarized in Table 3

RESULTS

Routine use of the 0–1 phage on clinical and culture material from different sources The results from routinely performed 0–1 tests on consecutive isolations from faecal specimens or on cultures submitted to the laboratory for identification were compared over a period of time chosen at random The comparison was comprised of (group I) 521 salmonella and 695 non salmonella strains tested at a laboratory section for the routine isolation of salmonella organisms from clinical specimens (group II) *215 salmonella and 52 non salmonella cultures* submitted to the National Salmonella Center and (group III) 2 260 *S paratyphi B* and *S typhi murium* cultures examined at a laboratory section for enteric phage typing The salmonella bacteria of the first and second group consisted of a variety of serological types

The 0–1 tests in the first two groups were performed in the same way an isolated colony from a culture was streaked on an Endo s agar plate (about 1 3 per cent agar) with a platinum loop and a loopful of undiluted phage suspension containing 10 –10^{10} plaque forming units (pfu) per ml was spotted on a closely streaked area of the plate The results were read after overnight incubation at 37 C In the third group undiluted phage suspension of the same strength as above was spotted on peptone agar plates (1 0 per cent agar) previously inoculated with overnight or actively growing broth cultures to give areas of confluent bacterial growth The results were read after five hours

A reaction characterized by no bacterial growth or less commonly by secondary growth within the area of the phage drop was classified as

positive Reactions with semi confluent lysis or isolated plaques were not found when undiluted phage suspensions were used An unbroken bacterial lawn sometimes with a slight thinning of the growth within the area of the phage drop was classified as a negative reaction

In the first group of strains chosen for the comparison a positive phage reaction was shown by 98 8 per cent of the Salmonella (Table 2) as opposed to 1 2 per cent by the non Salmonellas The corresponding percentages for the cultures of group II were 75 4 and 5 8 In group III 99 9 per cent were 0–1 positive The agreement between the results of the serological typing and of the 0–1 test thus was satisfactory for the strains examined at the routine diagnostic and phage typing sections but poor for the strains examined at the Salmonella Center

TABLE 2

Results from Routine 0-1 Phage Tests on Materials from Three Different Sources

Sources of cultures	Number of cultures						
	Total	Salm	Pos phage react	%	Non Salm	Pos phage react	%
I Diagnostic routine lab	1 216	521	515	98 8	695	8	1 2
II Salmonella Center	267	215	162	75 4	52	3	5 8
III Phage typing ref lab	2 260	2 260	2 258	99 9	0	–	

Enteric gram negative rods

TABLE 3

Comparison of Results Obtained with Different Techniques and Phage Concentrations on Material from Group II in Table 2

Technique and phage conc	Number of cultures					
	Salm	Pos phage react	%	Non Salm	Pos phage react	%
Endo s agar Phage titer c 10^9	215	162	75 4	52	3	5 8
Phage agar Phage titer 2×10^{11}	215	214	99 5	52		9 6

These figures for group II in Table 2 are included for comparison

Importance of phage concentration and test technique The strains routinely tested at the Salmonella Center were retested using the media and spot technique described under "Materials and Methods" The titer of the phage suspension used was now considerably higher about 2×10^{11} pfu/ml The multiplicity of infection in the spots was about 7 000

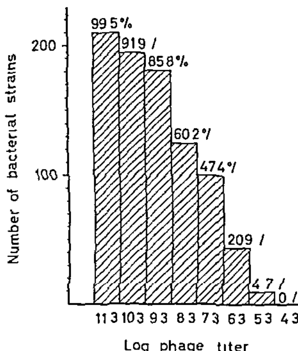


Fig 1

Spot test on 211 Salmonella strains
Distribution of positive reactions with various concentrations of phage 0-1

(Table 1) Only one of the salmonella strains (101/60 *S infantis*) was found to be *0-1* negative (*Table 3*) On the other hand the percentage of positive reactions among the non salmonellas had increased from 5 8 to 9 6

The plaque size is known to be inversely related to the agar concentration The different agar concentrations used for the Endo s agar plates at the Salmonella Center and for the plates employed for the specially adopted technique might be partly responsible for the difference in the results shown in Table 3 Thus is was found that five times as high a phage concentration was necessary to produce the same reaction on Endo s medium containing 1 6 per cent agar (plaque diameter about 0 4 mm) as on 1 0 per cent nutrient agar or Endo s agar (plaque diameter about 0 8 mm)

To study the sensitivity of the strains to various concentrations of 0-1 phage 211 of the originally available 215 strains sent in to the Salmonella Center were examined by spot tests of ten fold dilutions of the phage suspension As demonstrated in Fig 1 the sensitivity to the phage was found to vary greatly At a phage concentration of 2×10^{10} pfu/ml 16 strains were negative in addition to the one negative at a ten times higher concentration Each ten fold dilution of phage caused a stepwise loss of the number of strains showing a positive reaction The most sensitive strains were lysed confluently by a phage dilution containing 2×10^{5} pfu/ml At high dilutions of the phage sus

pension isolated plaques of about the same size (about 0.8 mm) as produced in the propagating B76 strain were observed in 107 (51 per cent) of the strains (here designated as strains of *reaction type I*). By counting the number of isolated plaques it was found that 25 strains had about the same high sensitivity to the phage as B76.

Irrespective of the phage dilutions no isolated plaques visible to the naked eye were found in the remaining 49 per cent of the strains (strains of *reaction type II*). All these strains were sensitive only to high phage concentrations, the most sensitive giving full positive reactions at a phage titer of 2×10^8. The reaction in some strains changed from positive to completely negative by one dilution step, the reaction in others changed more gradually on dilution of the phage by exhibiting a reaction characterized by an irregular inhibition of the bacterial growth within the area of the phage drop.

According to the findings of *Cherry et al.* (1954) a high percentage of resistance to phage 0-1 was found among strains classified in group E of the Kauffmann-White scheme. In the present material the difference in serological characteristics between the strains lysed by plaques and those affected differently is analyzed in Table 4. In all the strains represented 57 different serotypes belonging to 15 0-groups or subgroups, the most common types being *S. typhi murium* and *S. montevideo* with 34 and 53 strains respectively. As seen in the table both reaction types occurred in cultures belonging to 7 of the 9 groups represented by more than one strain. Both reaction types were found also

TABLE 4
Distribution of Serological Groups of Salmonella Strains of Varying Sensitivity to Phage 0-1

Serological groups	Number of strains belonging to		Total number of strains
	reaction type I	*reaction type II*	
A	7		7
B	30	34	64
C 1	42	38	80
C 2	8		8
C 3		1	1
D 1	6	10	16
D 2	1	1	2
E 1	5	13	18
E 4	1	5	6
F	3	1	4
H	1		1
I	1		1
L	1		1
M	1		1
Y		1	1
Total	107	104	211

According to the Kauffmann-White scheme

among most of the individual sero types However all 8 strains of *S enteritidis* belonged to reaction type II At least 4 of them were epidemiologically unrelated

Plaque formation in agar layer The inability of the virus to produce plaques observed in spot tests on the strains influenced only at high multiplicities was further confirmed by experiments in which broth cultures grown for four hours were mixed with equal volumes of 0–1 phage diluted in ten fold steps to 10^{8} The titer of the stock suspension was 10^{10} pfu/ml Six cultures of reaction type II were tested (five sero types *S typhi murium S montevideo S enteritidis S cholerae suis* and *S bareilly)* The mixture of phages and bacteria were plated by the standard agar layer method No plaques were observed When undiluted phage suspension or a dilution 1 10 was used only scanty or no bacterial growth was observed in contrast to the abundant *growth at higher phage dilutions No plaque formation was revealed even when* two fold dilution steps were made in the range between the concentrations lysing the bacteria or inhibiting the bacterial growth and the concentrations of phage which had no obvious influence on the bacteria

Effect of a phage free lysate and a purified phage preparation A high titer phage suspension was passed through a membrane filter (Gottingen *ultrafein* porosity *35 100 mμ) The undiluted phage free filtrate was spotted on cultures on the pro* pagating B76 strains and on five strains of reaction type II The spot test was negative for all strains including the very sensitive B76 A phage preparation purified by a two phase system (dextran methylcellulose *Albertsson* 1960) and containing 5×10^{11} pfu/ml was found to cause positive reactions in all six of these strains

Bacterial survival and growth after phage adsorption A series of experiments indicated that the 0–1 phage was adsorbed also to salmonella bacteria of reaction type II even to the only spot test negative salmonella strain found in the present study (101/60 *S infantis*) In some of these experiments phage adsorption was followed for periods up to 21 hours at 37 C by assay of the unadsorbed phage The finding of continuously diminishing number of free particles verified the inability of these strains to propagate phages

TABLE 5

Per Cent Surviving Bacteria after Adsorption of Phage 0–1

Strain		Per cent survivors
B76	(*S paratyphi B*)	1
8[illegible]/61	(*S bareilly*)	44
88/61	(*S muenster*)	57
98/61	(*S muenster*)	57
183/61	(*S typhi murium*)	3
2132/61	(*S enteritidis*)	41
101/60	(*S infantis*)	[illegible]9

Means of duplicate experiments

Adsorption mixtures Equal volumes of a pr armed 0–1 suspension (6×10^{10} pfu/ml) + a 4 hour Salmonella broth *culture* ([illegible]5 × 10^{8} *cells/ml*) Incubated 10 minutes at 3 C and diluted 10^{5} Unadsorbed phages were neutralized by adding 0 2 ml of the dilution to 0 8 ml anti-0 1 serum 1 50) and incubating at 37 C for 5 minutes 0 5 ml were then spread on an agar plate and colonies counted after incubation at 3 C

Control mixtures Equal volumes of the Salmonella broth culture and nutrient broth treated in the same ways as the adsorption mixtures

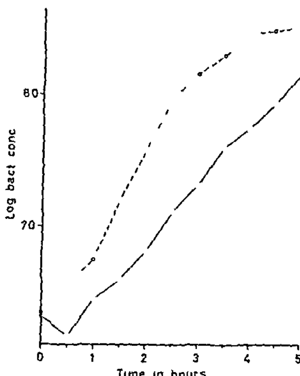


Fig 2

Crowth curve of an 0–1 negative Salmonella culture in the presence of the 0–1 phage
Phage 0–1 + bacterium mixture (× —— ×) One part washed cells from an over night broth culture of 101/60 *S infantis* suspended in broth (2 × 10 cells/ml) + nine parts of a broth dilution of purified phage 0–1 (8 × 10^{10} particles/ml) In cubation in a waterbath at 37 C. At each time interval samples of 0 1 ml were appropriately diluted 0 5 ml of the dilutions were mixed with agar and poured on agar plates for colony counting after incubation at 37 C overnight.
Control mixture (○ – – ○) One part bacterium suspension + nine parts of a broth dilution of purified coli phage T ? treated in the same wa as above

The relative resistance of strain 101/60 and five strains of reaction type II compared to strain B76 of reaction type I was demonstrated in an experiment where the bacteria were infected with a multiplicity of 240

As seen in Table 5 only one per cent of the B76 survived after the adsorption time and phage input employed whereas the survival of the bacteria belonging to reaction type II was considerably higher 29 to 57 per cent

Although the spot test on strain 101/60 was registered as negative (see Table 3) it was found that the bacterial growth after five hours incubation was thinner over *the area corresponding to the phage drop than over the surrounding area* Since previous results suggested that the phage adsorbed to this bacteria which resulted in a decrease in the number of viable organisms (Table 5) the bacterial growth in the presence of excess phage (40 000 phages per bacterium) was studied further for the first five hours of incubation (Fig ?)

An initial drop in the cell count was noted but this decrease was followed by a phase of rapid growth During the period studied the concentration of living cells in the phage bacterium mixture was at all times lower than the concentration in the control culture Thus it was found that the only salmonella strain from the Salmonella Center remaining negative in the spot tests in fact was affected also by the 0–1 phage

DISCUSSION

The present investigation shows that the concentration of the phage is an important variable if the 0–1 phage is to be used as an additional aid in routine salmonella identification To obtain almost 100 per cent positive reactions on salmonella strains a high phage input and a low agar concentration are necessary However these measures also will increase the number of positive reactions in cultures not belonging to the salmonella genus Furthermore the agar concentration in *e g* Endo s agar medium cannot be decreased to a level ideal for plaque formation if the bacterial colonies are to be used for slide agglutination tests In such a case the agar concentration has to be high enough to allow the formation of sufficient 0–antigens in relation to H–antigens When as a matter of convenience the 0–1 test is performed on the same agar plate as used for the routine morphological and serological identification of salmonella the choice of agar concentration has to be a compromise

The discrepancy between the occurrence of 0–1 positive non salmonella strains in the material from the different sources 1 2 against 5 8–9 6 per cent may be due to chance considering the small number of strains from the Salmonella Center but may also be due to some form of selection in this group Since the 0–1 phage is used as an additional diagnostic test at many laboratories certain serologically negative or doubtful cultures are forwarded to the Salmonella Center for further examination mainly because they were found to be 0–1 positive Some salmonella strains are indeed tracked down in this way but many cultures prove not to be Salmonella However the occurrence of 0–1 positive cultures not classified as Salmonella has not been reported as a serious disadvantage from other Swedish laboratories The information collected about the test in the routine diagnosis of salmonella organisms seems to justify a continuation of its use as an *additional* tool provided that a standardized technique is employed

The existence of a phage capable of adsorbing to almost all salmonella bacteria suggests the presence of a common structure in the cell walls and provides a tool for comparative investigations of the cell wall composition by studies of the receptor (*Lindberg* 1967)

In about half the number of the salmonella strains from the selected material investigated phage adsorption killed or inhibited the growth of the cells without signs of virus synthesis whereas in the other strains phage adsorption was found to result in an ordinary lytic cycle with synthesis of new virus particles Investigations on the cause of this

difference (Kallings & Lindberg 1967) indicate that the relative resistance to phage 0-1 may be due to the presence of prophage in the bacteria

SUMMARY

A comparison has been made between the results from the routine use of the 0-1 phage spot test on about 3 000 salmonella strains and about 700 other enterobacterial strains at three laboratory sections working with cultures from different sources and using different techniques (routine salmonella diagnosis salmonella reference identification and enteric phage typing) In the order of the sections listed positive phage reactions were obtained in 98 8 75 4 and 99 9 per cent of the serologically verified salmonella strains

When the strain material showing only 75 4 per cent positive reactions was retested with a high titer phage preparation and a special standardized technique 99 5 per cent positive reactions were obtained It was found that plaques were produced in 51 per cent of these strains which were lysed by low phage inputs No plaques were observed in the remaining 49 per cent of the strains They were only affected by high multiplicities of infection but did not support phage propagation

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RESISTANCE TO FELIX 0–1 PHAGE IN SALMONELLA BACTERIA

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Received 28 xii 66

The bacteriophage 0–1 capable of acting on most salmonella bacteria has been found to separate the salmonella strains into two groups (1) strains lysed by low phage concentrations and supporting the propagation of new particles and (2) strains influenced only at high phage concentrations but unable to produce phages (*Kallings* 1962 1967) The purpose of the present paper was to study the cause of the different action of the phage As the 0–1 phage particles were found to be adsorbed by the salmonella organisms influenced only at high phage concentrations the inability to produce 0–1 phages was assumed to be due to the presence of prophage In the present paper experiments are reported concerning the isolation of temperate phages and attempts to transform sensitive strains to resistant ones by means of these phages

MATERIALS AND METHODS

Bacteria Salmonella strains were identified by common biochemical and serological procedures All strains were isolated from clinical specimens and stored less than two years in sealed agar stab cultures Unless otherwise stated the bacteria were grown in broth for four hours at 37 C.

Phage 0 1 phage (*Felix & Callow* 1943) was propagated on strain B 76 (*S paratyphi B*) by means of the agar layer method (*Adams* 1959) Lysates sterilized by filtration through Jena glass filters and stored undiluted at +4 C were used as stock suspensions

Temperate phages isolated from lysogenic cultures were propagated on *S typhimurium* strains by the same method and stored under same conditions

Media The same Difco nutrient agar and broth described in a previous paper (*Kallings* 1961) was used In addition the synthetic medium M 9 (*Adams* 1959) was used

Diluents Nutrient broth or phosphate buffered saline pH 7 4 was used as diluents

Induction The bacteria were adapted to grow in M 9 medium at 37 C. Subcultures in M 9 were grown at 37 C for four hours A shallow layer of the culture in a Petri dish was exposed for 10 seconds to ultraviolet light (2537 Å) at a distance of 50 cm with continuous shaking Afterwards the bacteria were incubated at 37 C for 30 minutes and centrifuged at 2000 g for 20 min

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Preliminary report given to the Medical Microbiology Division of the Swedish Medical Society 1967

With the technical assistance of Mrs *Maud Lindberg* and Mrs *Gerd Lundström*

Phage assay The phage concentration was measured by plaque counting or the spot technique described previously (*Kallings* 1967)

Antiphage sera Hyperimmune sera against phage 0-1 and phages 105 35 27 88/61 and 250/61 were produced by injecting rabbits subcutaneously with stock phage suspensions The time schedule was 2 injections per week of 5 ml each for 4 weeks followed by 5 ml twice after another two weeks The sera were inactivated at 56 C for 30 min

Neutralization test A mixture of 0 5 ml phage dilution (containing about 1 0 × 10^3 pfu/ml) and 0 5 ml diluted antisera were incubated at 37 C for 15 minutes in a water bath From this mixture 0 5 ml were mixed with 4 0 ml soft melted agar at 45 C plus 0 5 ml of a bacterial culture 2 ml were plated on an agar base and incubated at 37 C overnight The degree of neutralization was determined by comparing the number of plaques formed with that of controls (phage + broth)

EXPERIMENTAL

Isolation of Phage 105 from a Lysogenic Salmonella Strain

Twenty *Salmonella typhi murium* cultures of different phage and fermentation types were tested for sensitivity to ten fold dilution steps of the 0-1 phage by the spot technique Readings were carried out after five hours incubation at 37° C Reactions without any evidence of phage multiplication and an unbroken bacterial lawn were classified as *negative* Reactions characterized by a clearly lysed area (i e no bacterial growth) semiconfluent lysis or isolated plaques within the area of the phage drop were considered as *positive*

Eighteen cultures were lysed supporting the propagation of new particles at low multiplicities of infection The two remaining cultures gave negative reactions at low multiplicities although influenced by high phage concentrations they were unable to maintain the propagation of the 0 1 phage One of these two cultures (S 105) was chosen for an attempt to isolate a temperate phage responsible for the resistance against phage 0-1

After induction of strain 105 the supernatant was tested against the 20 S *typhi murium* strains by the spot technique An isolated plaque on one (S 204) of the strains lysed was selected By repeated isolations and subcultures of single plaques on the strains S 204 a phage suspension labelled 105 with a titer of about 1 0 × 10^9 pfu/ml was prepared

The phage was again tested against the 20 S *typhi murium* strains as above All strains but strain 105 were now lysed

No contaminating phages originating from the propagating strain (S 204) of phage 105 could be demonstrated when the strain was induced and the supernatants tested against the 20 indicator cultures.

Conversion to 0 1 Resistance by the Phage 105

Phage 105 was spotted on the 18 strains lysed by phage 0-1 After prolonged incubation isolated colonies growing within the area originally lysed confluently were picked up and identified as *S typhi murium* The bacteria were then tested for sensitivity to phage 0 1 by spot technique Resistant bacteria were found in 8 of the 18 cultures

These bacteria were affected only at high multiplicitiees of infection (without plaque formation)

To check if the 8 *S typhi murium* cultures were lysogenized by phage 105 the cultures were induced with ultraviolet light The supernatants were tested against the 20 *S typhi murium* indicator strains by the spot test Strong reactions confluent or semiconfluent lysis appeared on all strains with the exception of strain 105

The phages obtained by induction of the 8 converted cultures were neutralized by hyperimmune serum against phage 105 (Table 1)

TABLE 1

Neutrali ati n of Phages Isolated from Lysogeni ed Salmonella Cultures by Antiserum aga nst Phage 105

Phag s or induced culture supernatant	Anti erum	Per cent neutralized
238	105	98 4
237	105	98 0
207	105	100
131	105	97 4
100	105	99 1
99	105	99 9
7	105	99 1
561	105	99 5
105	105	100

Dilution of antiserum against phage 105 1 250

The neutralization of phages derived from lysogenize l cultures ranged between 98 and 100 per cent With two exceptions unneutralized phage particles gave rise to plaques with the same morphology as those caused by phage 105 The two exceptions were from the supernatants of the cultures 237 and 238 which in addition to plaques with the characteristic size and shape of phage 105 yielded larger plaques with a more pronounced turbid center The unneutralized phage particles could be either phage 105 which had not been neutralized or other phages released from the various *S typhi murium* cultures during this last induction To investigate this possibility the two different plaque types were cut out propagated and use l in a new neutralization te t with h perimmune serum against phage 105

The turbid plaques from supernatants 237 and 238 yielded phages which were not inactivated by hyperimmune serum against phage 105

Inability to Propagate Phage 0–1 on the Lysogenized Cultures

0–1 phage was used to infect actively growing cultures of the 8 lysogenized *S typhi murium* strains at a concentration of phage which used to cause semiconfluent lysis on the unconverted bacteria The experiment was designed to see if phage 0–1 could multiply within these bacteria

Table 2 shows that salmonella strain 105 which yielded the temperate phage 105 was unable to maintain the propagation of phage 0–1 As a comparison strain 201 the propagating strain of phage 105 gave rise to about a 400 fold increase in phage 0–1 titer The phage was unable to

multiply also on the lysogenized cultures 99 and 561 and gave a small rise in titer on the other 6 cultures.

TABLE 2
Propagation of Phage 0-1 on the Lysogenized Cultures

Strain	Original 0-1 titer (pfu/ml)	Resulting 0-1 titer (pfu/ml)	Difference in multiples
105	1.0×10^8	4.6×10	− 2
238	3.4×10^8	1.5×10^9	+ 4
237	3.4×10^8	1.0×10^9	+ 4
207	3.4×10	4.5×10^8	+14
131	3.4×10^8	2.6×10^9	+ 8
100	3.4×10^8	1.2×10^9	+ 4
99	3.4×10^8	3.0×10^8	± 0
7	3.4×10^8	1.2×10^9	+ 4
561	3.4×10^8	2.7×10^8	− 1
204	1.1×10	3.9×10^9	+390

The slight amount of phage 0-1 propagation in some of the lysogenized strains may be due to a small portion of the bacterial population which do not contain prophage. Phage 0-1 propagated on the unconverted indicator strains gave 30-1300 folded rises in titers.

Isolation of other Temperate Phages Causing Resistance to Phage 0-1

To investigate the frequency of lysogeny causing 0-1 resistance 34 Salmonella strains (belonging to 10 serotypes) lysed only by high 0-1 phage concentrations without phage multiplication were induced with ultraviolet light and treated as above. The supernatants were plated on the 20 *S. typhi murium* cultures earlier used as indicator strains by the spot technique to determine the presence of phages. Four strains were found to harbour phages which produced large turbid plaques which were different from the phage 105 plaques. Two of these phages were capable of converting three of the indicator strains to 0-1 resistance. The strains harbouring phages with converting ability belonged to serogroup E_1: *S. vejle* (235/61) and *S. muenster* (88/61). The phage from *S. muenster* was capable of converting two strains while the phage from *S. vejle* converted one. In this experiment nonconverting phages were obtained from a *S. typhi murium* (27/61) serogroup B and a *S. montevideo* (250/61) serogroup C_1.

The three converted cultures were induced in order to investigate if they were lysogenized with phage 235/61 or 88/61. Supernatants were tested in a spot test against the 20 S. *typhi murium* indicator strains. Positive reactions (mostly less than semiconfluent lysis) appeared on 18 strains, this time including strain 105. Hyperimmune serum prepared against the two phages neutralized between 9[illegible] and 100 per cent of the phages in the supernatants.

Serological Relationships between Phage 0–1 Resistance Converting and Non Converting Phages

The converting phages 105, 235/61, 88/61, nonconverting phages 27/61, 250/61 and phage 0–1 were compared in a neutralization test.

TABLE 3

Crossreactions in a Neutralization Test between Phage 0–1 Immunity Converting and Non Converting Phages

Anti Sera	Serum dilution	0–1 phage	Converting phages 105	235/61	88/61	Nonconverting 27/61	250/61
0–1	1:1000	+++	—	—	—	—	—
105	1:250	—	+++	—	+	—	+
235/61	1:100	—	+	++	+	+	+
88/61	1:100	—	—	++	+++	++	+++
27/61	1:50	—	—	++	+++	+++	—
250/61	1:50	—	—	+++	+++	+++	+++

+++ complete neutralization
++ 50–99 per cent neutralization
+ 25–49 per cent neutralization
— < 25 per cent neutralization

Except for phage 0–1, anti-0–1 serum did not inactivate any of the investigated phages. Serum against phage 105 gave weak cross reactions with phages 88/61 and 250/61 while serum against phage 235/61 gave a weak reaction with phage 105. Cross reactions were frequently detected within the temperate phages 235/61, 88/61, 27/61 and 250/61.

CONCLUSION

As has been shown in a previous paper (*Kallings* 1967) only about 50 per cent of salmonella strains belonging to different serotypes were able to support phage 0–1 propagation. However, the majority of the other 50 per cent were found to adsorb the 0–1 phage although the virus did not multiply. In the present paper this inability of phage multiplication in spite of adsorbtion was shown to be due to the presence of prophage in some strains isolated from clinical cases of salmonellosis. The temperate phages were isolated and used to convert 0–1 sensitive salmonella strains into 0–1 resistant ones.

Temperate phages isolated from one salmonella serogroup (E 1) were able to convert bacteria of another serogroup (B) to 0–1 resistance. The converting phages isolated from different sources were not identical as judged by plaque morphology and serology. However, a certain serological relationship was demonstrable. The converting phages were as well serologically related to non converting phages isolated from other lysogenic salmonella cultures. None of the phage isolated, whether con

verting or not was serologically related to the 0–1 phage The broad antigenic relationship of various phages isolated from lysogenic salmonella cultures is notable

SUMMARY

Although many salmonella cultures were lysed by high concentrations of 0–1 phage they could not support virus multiplication This was shown to be due to the presence of prophage Resistant bacteria were induced to release temperate phages capable of converting 0–1 susceptible salmonella strains to resistant ones

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AN ANAEROBIC ORAL FILAMENTOUS ORGANISM POSSIBLY RELATED TO *LEPTOTRICHIA BUCCALIS*

2. *Composition of Cell Walls*

By

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Received ?6.i.67

In a previous paper some morphological, physiological and serological data of an anaerobic oral filamentous organism were described (9). The organism had some properties in common with *Leptotrichia buccalis* but differed from that species in showing a heterofermentative type of sugar metabolism. In addition, little or no serological cross reactivity was found between the filamentous organism and typical *L. buccalis* strains.

The present paper deals with the chemical composition of cell walls from the atypical filamentous organism and from *L. buccalis*.

METHODS

Preparation of Cell Walls

Cell walls were prepared from the following filamentous strains: L? L23, L44, L49 and L52, and from the *L. buccalis* strains L11 and L66. The strains, which were also included in the previous study (9), were grown in nutrient broth enriched with 0 1 per cent cysteine hydrochloride, 0 3 per cent yeast extract (Difco), 1 per cent glucose and 5 per cent of human ascitic fluid. After ? to 4 days incubation at 37 C, the bacteria were harvested by centrifugation, washed in saline and kept frozen until used.

The procedures employed for preparation of the walls were essentially as described by Yoshida *et al.* (15). The density gradient centrifugation was carried out at 900 × G for 1 hr. The washed walls were stored in the freeze dried state.

Trypsin digestion of the prepared walls was performed with crystalline trypsin (Trypure Novo) freshly prepared in 0 0? M tris buffer of pH 7 8 using 0 1 mg trypsin per mg walls. The digestion was carried out at 37 C for ?0 hrs.

Paper Chromatography

Hydrolysis was performed in sealed tubes in 0 1 N HCl and 3 N HCl for ? and 3 hrs at 100 C, respectively, and in 6 N HCl for 18 hrs at 105 C. The d[illegible]d materials were made up in distilled water and subjected to circular chromatography using Whatman paper No. 1.

Developing Solvents: (A) *n*-Butanol : acetic acid : water (4 : 1 : 1) and (B) phenol : water (4 : 1 w/v) for amino acids and amino sugars. (C) *n*-butanol : pyridine : water (6 : 4 : 3) for neutral sugars. (D) *n*-propanol : ammonia (d 0 91) : water (6 : 3 : 1) for

sugar alcohols and (E) iso propanol 2 N hydrochloric acid (65 35) for purine and pyrimidine derivatives

Spray reagents Amino acids were detected with 0 4 per cent ninhydrin in acetone containing 2 per cent acetic acid and aldoses with aniline hydrogen phthalate in water saturated *n* butanol The Elson Morgan reagent as modified by *Partridge* was used for detection of amino sugars Sugar alcohols were examined for by sodium periodate benzidine and proline and hydroxyproline by 5 per cent isatine in water saturated *n* butanol containing 4 per cent acetic acid

Quantitative Analyses

The amino acid content in 6 N HCl hydrolysates of undigested and trypsin treated walls was determined by the ninhydrin method essentially as described in (10) A solution of equal parts of glutamic acid alanine and diaminopimelic acid was used as standard

Hexosamines were determined by the method of *Rondle & Morgan* (11)

Estimation of neutral sugars was performed with α naphthol as described in (10) Glucose was used as standard

The sulphuric acid cysteine reaction of Dische (9) was used *for the detection and quantitative determination of heptose* Standard samples of different aldoheptoses were kindly supplied by Dr N K *Richtmyer* National Institutes of Health Bethesda Md

The sulphuric acid cysteine reaction was also used for *determination of rhamnose* (3)

Lipid was estimated by determination of lipid ester groupings as described by *Snyder & Stephens* (14)

EXPERIMENTAL PROCEDURES AND RESULTS

Almost complete disruption as judged by Gram staining was obtained by passing the frozen bacteria twice through the X press (see 15) The disintegrated material tended to clump even after removal of the protoplasm fraction and repeated washings The cell wall layer in the middle of the sucrose gradient tube was therefore very diffuse and considerable amounts of walls were lost in the sediment which contained the few whole cells

The freeze dried wall preparations varied in colour from white to light grey The preparations were free from nucleic acids as no purine or pyrimidine bases were found when 0 1 N hydrochloric acid hydrolysates were examined by paper chromatography

Digestion with trypsin was carried out on wall preparations from strains L14 L49 and L11 Following this treatment the preparations lost two thirds in weight varying somewhat from one batch to another The freeze dried trypsin digested walls were white and contrary to the undigested walls homogeneous suspensions were easily obtained with water or buffers

Paper Chromatography

The amino acids and neutral sugars found in hydrolysates of undigested and trypsin digested walls are listed in Tables 1 and 2 respectively While the filamentous organism and *L buccalis* contained the same amino acids in their walls the filamentous organism differed clearly from *L buccalis* with respect to cell wall sugars

TABLE 1

Amino Acids in 6 N Hydrochloric Acid Hydrolysates of Cell Walls from the Filamentous Organism (5 Strains) and L. buccalis (2 Strains)

	Undigested walls	Trypsin digested walls
Alanine	++	+++
Glutamic acid	++	+++
Diaminopimelic acid	++	+++
Glycine	++	++
Lysine	++	+
Serine	++	+
Valine	++	+
Leucine	++	+
Threonine	++	+
Aspartic acid	++	—
Proline	+	—
Methionine	+	—
Arginine	+	—

In addition traces of a slow moving component were seen in hydrolysates of *L. buccalis* run in system (B)
+++ ++ + Relative amounts judged by visual comparison of the colour intensity on ninhydrin treated chromatograms
— Not detected

TABLE 2

Neutral Sugars in 3 N Hydrochloric Acid Hydrolysates of Cell Walls from the Filamentous Organism (5 Strains) and L. buccalis (2 Strains)

	Filamentous organism		*L. buccalis*	
	Undigested	Trypsin digested	Undigested	Trypsin digested
Glucose	+++	+++	+++	+++
Galactose	—	—	+	+
Rhamnose	++	++	—	—
Heptose	++	—	—	—

In addition traces of arabinose were found in undigested walls from *L. buccalis*
+++ ++ + Relative amounts judged by visual comparison of the colour intensity of aniline hydrogen phthalate treated chromatograms
— Not detected

Glucosamine and muramic acid were found in undigested and trypsin treated walls from all strains Sugar alcohols were not detected in 3 N hydrochlorid acid hydrolysates of undigested walls

Nature and Location of the Heptose in the Cell Wall

The sugar listed as heptose in Table 2 gave the same red brown colour with aniline hydrogen phthalate as standard samples of aldo heptoses It moved like D glycero D galacto heptose in the solvent systems (B) and (C)

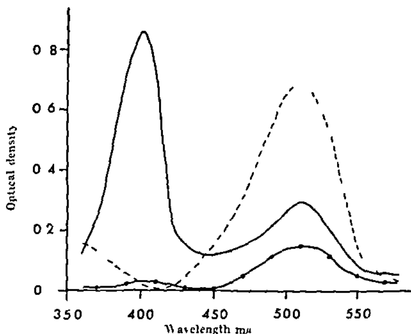


Fig 1

Dische reaction spectra given by ——— 500 μg cell walls from the filamentous organism strain L44 ●—● the isolated heptose read against a paper blank from the same chromatogram ----- 100 μg D glycero D galacto heptose

In order to confirm this chromatographic finding the assumed aldo heptose was isolated and subjected to the sulphuric acid cysteine reaction of *Dische* The isolation was performed in the following way an evaporated 3 N hydrochloric acid wall hydrolysate (strain L44) was made up in distilled water and passed through a column of Amberlite IR–120 in the hydrogen form While the amino acids and amino sugars were retained by the resin the neutral sugars went straight through the column The fractions containing the sugars were evaporated *in vacuo* re dissolved in a small volume of distilled water and subjected to paper chromatography in solvent system (C) After having traced the different sugars by treating small radiate strips of the dried chromatogram with aniline hydrogen phthalate the heptose was eluted from the paper with distilled water

The absorption spectra of the chromogens produced in the *Dische* reaction by the isolated heptose and a sample of undigested walls from strain L44 are shown in Fig 1 The high optical density at 400mμ produced by the wall sample is due to rhamnose and glucose which produce absorption maxima at 400 and 410 mμ respectively

The aldoheptose was only found in minimal amounts in hydrolysates of the protoplasm fraction of the bacterial cells (the supernatant fluid following the first centrifugation of the crushed suspension) and could not be detected in hydrolysates of the uninoculated medium used for

cultivation of the bacteria. Free heptose was not found in the supernatant after trypsin digestion but was readily demonstrated in hydrolysed samples of it together with amino acids and traces of glucose.

In another experiment whole cells from strain L44 were treated with trypsin. The treated cells were Gram negative. Some cells were slightly swollen but with clear margins and cell fragments or other evidence of disintegration were not found. The trypsin digested cells were then hydrolysed by boiling in 3 N hydrochloric acid for 3 hrs and the evaporated hydrolysate examined for neutral sugars by paper chromatography. Only traces of heptose were demonstrated while a strong heptose spot was obtained with a hydrolysate of whole cells from strain L44 treated with tris buffer.

The experiments performed show that the aldoheptose is part of an outer cell wall layer.

Quantitative Analyses

The results of the quantitative analyses have been compiled in Table 3. The findings varied somewhat from one batch to another, in particular the values obtained for amino acids and lipid in the undigested walls. In a few preliminary experiments the lipid content of the undigested walls from strains L44 and L49 was estimated by ether extraction of hydrolysed wall samples (13). The lipid content estimated by this method varied between 8 and 16 per cent. In order to give reproducible results this method required large amounts of walls (more than 20 mg each sample). The micromethod of *Snyder & Stephens* was therefore used in the further experiments.

D-glycero-D-galacto-heptose was used as standard in the *Dische* reaction.

As shown in Table 3 the wall preparations from the filamentous organism contained more sugar but less amino acids than the walls from *L. buccalis*.

TABLE 3

Quantitative Analyses on Cell Walls from the Filamentous Organism and L. buccalis (Per Cent of Dry Weight)

		Filamentous organism				*L. buccalis*	
		L44 u	*L44 t*	*L49 u*	*L49 t*	*L11 u*	*L11 t*
Amino acids		52	28	58	[illegible]	70	46
Neutral sugars	(as glucose)	20	35	23	4	10	21
Rhamnose		5	19	4	1		
Heptose		7		6			
Hexosamines	(as glucosamine)	3	5		4	5	7
Lipid	(as tripalmitin)	21	4	18	3	13	5

The values listed are mean values from examinat[illegible] two or three different batches.

No correction has been made for water uptake during hydrolysis.

u = undigested walls, t = trypsin digested [illegible]

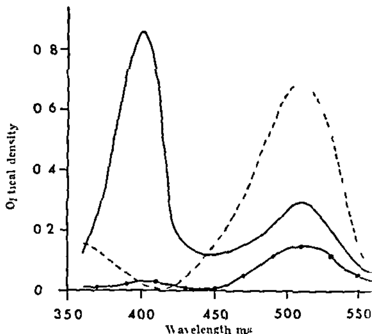


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DISCUSSION

The difficulties in obtaining a distinct cell wall layer in the density gradient tube and the varying results obtained by the quantitative analyses indicate that the undigested walls were rather heterogeneous and crude preparations On the other hand they were free of nucleic acid

Discrepant figures for cell wall lipid were obtained by ether extraction of hydrolysed samples in preliminary experiments and by estimation through the determination of carboxylic acid ester groups This may in part be due to some hydrolysis of the lipid with loss of certain groups by the first mentioned method The micromethod of *Snyder & Stephens* has been used with success in quantitative examinations on endotoxins (7) but as far as I know has never been used for determination of lipids in bacterial cell walls The possibility that this method includes groupings in the complicated cell wall structure other than the lipid esters cannot be excluded

With the above reservations in mind it is nevertheless safe to conclude that the walls of the strains examined in addition to the cell wall glycopeptide or mucopeptide contain both polysaccharide protein and lipid

The demonstration of an aldoheptose in the walls of the filamentous organism is interesting Aldoheptoses have up to now only been found as constituents of polysaccharides extracted from Gram negative bacteria The heptose found in the filamentous organism is without doubt located in the outer part of the cell wall It could be removed almost *quantitatively by treating whole cells with trypsin and this did not* cause any gross alterations in cell morphology Similar treatment of the isolated walls removed in addition large amounts of protein and lipid (cf Table 3) Large amounts of protein and lipid together with smaller amounts of polysaccharide were also removed by treating the *L buccalis* cell walls with trypsin This indicates that the filamentous organism and *L buccalis* may contain an outer lipo protein layer connected with some polysaccharide

The finding of the same amino acids in cell walls of the filamentous organism as in *L buccalis* strengthens the view put forward in the previous paper that the filamentous organism represents a new *Leptotrichia* species This view is not challenged by the demonstration of a different sugar pattern in the walls of the two species Numerous studies on cell walls have shown that the pattern of amino acids tends to characterize taxa higher than species and that the nature of the sugars (and amino sugars) characterizes species

It was hoped that the chemical characterization of the isolated cell walls also would provide information on the classification of the genus *Leptotrichia* Most authors are now apt to include this genus in the family *Lactobacillaceae* tribe *Lactobacilleae* (5 12) The isolation of

endotoxic lipopolysaccharides from *L buccalis* (1 6) raises doubts about this view The finding in the present study of lipid and a low amino sugar content in both undigested and trypsin treated walls is in agreement with previous studies on cell walls from Gram negative species The demonstration of a few major amino acids in the trypsin treated walls however points more to the Gram positive bacteria Lipid is known to occur in mycobacteria and corynebacteria and has also been found occasionally in cell walls from streptococci (8) The very low amino sugar content may to some extent be due to incomplete hydro lysis in the *Rondle & Morgan* s reaction (4) Further studies are needed to clarify the taxonomical position of genus *Leptotrichia*

SUMMARY

The chemical composition of undigested and trypsin digested cell walls from an anaerobic oral filamentous organism and from *Leptotrichia buccalis* have been compared Undigested walls from both organisms contained polysaccharide protein and lipid The walls from the two organisms contained the same amino acids Glucose and smaller amounts of galactose were found in *L buccalis* cell walls and glucose rhamnose and an aldoheptose possibly D glycero D galacto heptose in the walls of the filamentous organism Treatment with trypsin removed great amounts of protein and lipid from the walls and in addition all the heptose found in undigested walls of the filamentous organism

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SEPARATION AND PARTIAL PURIFICATION OF THE SIXTH, SEVENTH AND EIGHTH COMPONENTS OF HUMAN HAEMOLYTIC COMPLEMENT

By

U Nilsson

Received 6 ii 67

Recent work has resulted in the resolution of the classical third component of haemolytic complement into six factors (*Nelson et al* 1966 *Wellensiek & Klein* 1964 *Müller Eberhard* 1965 *Nilsson & Müller Eberhard* 1966 *Polley & Muller Eberhard* 1966 b) In accordance with a recently proposed nomenclature (Complement Workshop La Jolla 1966) they will here be designated C 3 C 5 C 6 C 7 C 8 and C 9 C 3 and C 5 in this sense are analogous to β_{1C} and β_{1F}-globulin respectively (*Nilsson & Muller Eberhard* 1965)

The main purpose of this paper is to report a procedure enabling separation and recognition of C 6 C 7 and C 8 as individual factors The distribution of C 6 C 7 and C 8 in fractions obtained by a recent procedure for isolating C 3 and C 5 (*Nilsson & Muller Eberhard* 1965) will also be described

MATERIAL AND METHODS

Starting material The euglobulin fraction was prepared from fre h hum n serum after dialysis against a low ionic strength EDTA buffer as described by *Polley & Müller Eberhard* (1967c) In brief the procedure comprises the f llo v ng steps a) extensive dialysis of approximately 1000 ml pooled fresh erum f m four individuals against 0 003 M EDTA pH 5 4 b) separation of the pr pi t-d euglobulins by centrifugation and decantation of the p eudoglobulin f t careful washing of the precipitate in the EDTA solution of pH 5 4 M = 0 005 d) dissolving of the precipitate in 20 ml phosphate buffer pH 7 0 M = 0 0 n ng 800 mg of NaCl e) ultracentrifugation for 1 hour at 26000 × G ed t remove the bulk of the lipids by flotation

Triethylaminoethyl (TEAE) cellulose chromatography wa p rf m d as described earlier (*Nilsson & Müller Eberhard* 1965) with some m i t at n Approximately 400 ml TEAE cellulose packed in a 3 × 70 cm lu n w equilibrated with

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0 03 M sodium phosphate buffer of pH 8 1 The euglobulin fraction was dialysed against the same buffer before application The elution procedure comprised a) an initial washing step with 1000 ml of 0 03 M sodium phosphate buffer pH 8 1 b) a gradient of increasing salt concentration and decreasing pH The gradient was prepared in an open cone sphere system *consisting of a 1000 ml round flask as a mixing* chamber and a 500 ml Ehrlenmeyer flask. The flasks were connected by a siphon Initially the round flask was filled with 0 03 M sodium phosphate buffer pH 8 1 and the Ehrlenmeyer flask with 0 25 M monobasic sodium phosphate c) washing with 500 ml 0 25 M monobasic sodium phosphate and d) a washing with 800 ml 0 7 M NaCl in 0 25 M monobasic sodium phosphate

Hydroxyl apatite chromatography was performed *according to the procedure of Tiselius et al* (1956) As described previously (*Nilsson & Müller Eberhard* 1965) sodium potassium phosphate buffers of pH 7 9 and of stepwise increasing concentration were utilized in the elution procedure Buffers of suitable concentrations as measured by their specific conductance per cm at 23 C, were obtained by mixing appropriate volumes of 0 0? M sodium phosphate and 0 65 M sodium potassium phosphate of pH 7 9

C 6 C 7 and C 8 were separated on 200 ml of loosel packed hydroxyl apatite in a 2 5 × 40 cm column The adsorbent was equilibrated with phosphate buffer of a conductance of 6 millimhos per cm and a pH of 7 9 Fractions containing C6 C7 and C 8 obtained by TEAE – cellulose chromatography were pooled and applied as such onto the hydroxyl apatite column This pool had a conductance of approximately 5 millimhos per cm and a pH of 7 5 to 8 The elution procedure comprised four subsequent steps of 600 to 800 ml phosphate buffer of a conductanc of 6 11 12 and 13 millimhos per cm respectively

For the separation of C 3 and C 5 400 ml of loosely packed hydroxyl apatite in a 3 × 70 cm column was utilized The adsorbent was equilibrated with a phosphate buffer of a conductance of 8 millimhos per cm A pool containing C3 and C5 obtained by TEAE chromatography was applied without preceding concentration or dialysis Its conductance was in the vicinity of 7 millimhos per cm and its pH around 7 5 Elution was done in four subsequent steps with 600 to 800 ml phosphate buffer of a conductance of 8 12 13 and 14 5 millimhos per cm respectively

The entire separation procedure was performed at +4 C As an additional precaution to prevent inactivation of the factors due to bacterial growth all buffers utilized contained 5 × 10 5 M chloramphenicol Furthermore all fractions were passed through millipore filters (Ø = 0 45 *µ*) before any of the chromatographic separation procedures concentration by ultrafiltration or storage at +4 C. Since chloramphenicol of high OD 280 was present in the buffers protein analysis was performed with the Folin method

Disc electrophoresis was performed according to *Davis* procedure (1964) Equal aliquots of each preparation were separated simultaneously on two polyacrylamide gel columns *One of the gels was stained for protein the other was cut in 0 1 inch* segments which were carefully minced in 0 5 ml gelatin veronal buffer (*Mayer* 1961) The eluates obtained in this way were analysed for haemolytic activity After correction for the difference in size of the stained and unstained gel haemolytic activity of the segments was correlated with the protein bands of the stained gel

Preparative electrophoresis was performed on Pevikon blocks according to *Müller Eberhard* (1960)

Sucrose density gradient ultracentrifugation was performed according to *Kunkel* (1960)

Haemolytic assay techniques Veronal buffered saline containing 0 1 per cent gelatin (Difco) (*Mayer* 1961) gelatin veronal buffer was utilized as diluent EAC 1a 4 2a 3 cells were prepared as described previously (*Nilsson & Müller Eberhard* 1967a) This cell antibody complement complex contains an oxidized form of C 2 which is approximately 10 times more active than the non oxidized form of the factor (*Polley & Müller Eberhard* 1966a) Haemolytic assays were performed in an 0 5 ml reaction volume containing 2 5 × 10 EAC1a 4o 2a,3 cells and EDTA in 0 01 M concentration

C5 activity was determined in human serum diluted 1 80 previously depleted of C3 C4 and C5 activity by treatment with 1 M KCNS (*Dalmasso & Müller Eberhard* 1966) C6 C7 C8 and C9 were assayed in mixtures of purified components lacking the factor assayed for C6 was also measured by reconstitution of C6 deficient

rabbit serum as described previously (Nilsson & Müller Eberhard 1965 Rother et al 1966) Highly purified human C 9 (Hadding et al 1966) for haemolytic assays was kindly supplied by Dr U Hadding As a qualitative screening procedure aliquots of chromatographic eluates were tested for reconstituting activity of haemolytic test mixtures deficient in single complement components Haemolytic assay procedures were also utilized for quantitation of C'5 C'6 C'7 and C'8 in serum serum fractions and purified preparations Purified components in aliquots containing 2 per cent human albumin (Behringwerke reinst) frozen in liquid N and stored at —70 C served as reference material Each reference preparation was assigned an arbitrary number of units per ml For each determination the unknown sample and a freshly thawed aliquot of the appropriate reference were diluted in twofold steps The haemolytic activity of both was determined under identical conditions in the desired test system The number of arbitrary units per ml of the unknown sample was derived from the relationship between the amounts of the test and the reference samples giving 50 per cent haemolysis

RESULTS

Partial Purification of C 6 C 7 and C 8

In addition to the bulk of the C 3 and C 5 (*Nilsson & Muller Eberhard* 1965) the euglobulin fraction of human serum was found to contain approximately 90 per cent of the C 6 15 per cent of the C 7 and 40 per cent of the C 8 haemolytic activity present in the original serum pool The degree of purification obtained by euglobulin precipitation was approximately 25 4 and 15 fold for C 6 C 7 and C 8 respectively In spite of the relatively low yield of C 7 the euglobulin fraction was considered a suitable source of C 6 C 7 and C 8 as well as of C 3 and C 5 since all five factors could be isolated from the same starting material As described previously (*Nilsson & Muller Eberhard* 1965) TEAE cellulose chromatography was useful as an intermediate isolation step for the two last mentioned components This procedure was also found to enable the separation of a portion of C 6 C 7 and C 8 from C 3 and C 5 A separation of euglobulin by TEAE cellulose chromatography is illustrated in Fig 1 The bulk of the IgG of the euglobulin preparation was eluted in fractions 10—25 in the initial washing step The protein eluted in fractions 165—200 at a pH of 6 8 to 6 3 and at a conductance of 6 to 7 6 millimhos per cm has previously been found to contain the bulk of C 3 C 5 and β_{1E} globulin (*Nilsson & Muller Eberhard* 1965)

Assays of the eluate for haemolytic activities revealed C 7 and C 8 in fractions 30 to 250 C 6 emerged in fraction 142 at pH 7 9 and at a conductance of 5 4 millimhos per cm and trailed into the area containing C 3 and C 5 As demonstrated by *Hadding et al* (1966) C 9 was eluted late largely separated from the other complement components Material suitable for the further separation of C 6 C 7 and C 8 was obtained from a pool of fractions 60 to 160 (pool II Fig 1) This material was free of C 3 C 5 and C 9 activity and was separated from the bulk of unrelated proteins of the euglobulin fraction A pool of fractions 167 to 197 (pool IV Fig 1) was selected for the isolation of C 3 and C 5 in the way described previously (*Nilsson & Muller*

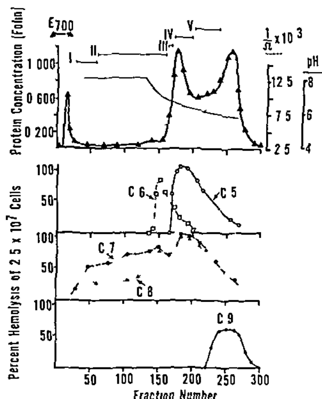


Fig 1

Distribution of C 3 C'5 C 6 C 7 C 8 and C 9 in euglobulin fractions after chromatography on TEAE-cellulose Gradient started at fraction 50 followed by washing with 0 05 M NaH phosphate at fraction 190 and by 0 7 M NaCl in 0 05 M NaH phosphate at fraction 240 The protein elution pattern is given in the top diagram The distribution of complement component activities is recorded in the three lower diagrams Roman figures indicate pools prepared from the eluate as discussed in the text Pool II was utilized for the further separation of C 6 C 7 and C 8 Pool IV for the further separation of C 3 and C'5

Eberhard 1965) The amounts of C 6 C 7 and C 8 of pools II and IV in relation to the total yield of these activities in the chromatographic eluate (pool I—V Fig 1) were estimated Pool II contained 40 per cent of the C 6 C 7 and C 8 activities respectively Pool IV contained 50 per cent of the C 6 activity and 30 per cent of the C 7 and C 8 activities

Hydroxyl apatite chromatography was utilized for the further separation of C 6 C 7 and C 8 (Fig 2) The column was equilibrated with a phosphate buffer of pH 7 9 and a conductance of 8 millimhos per cm Pool II (Fig 1) was concentrated by pressure dialysis against the same buffer and subsequently applied to the column Elution was performed in four subsequent steps with phosphate buffers of pH 7 9 and of stepwise increasing salt concentration Following application of the material the adsorbent was washed with a buffer of a conductance of 8 millimhos per cm Some protein unrelated to the complement

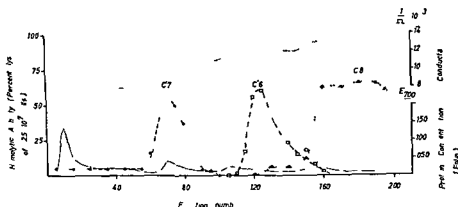


Fig 2

Chromatographic separation of C6 C7 and C8 on hydroxyl apatite The protein (Pool II Fig 1) was applied in phosphate buffer pH 7 9 conductance 8 millimhos/cm It was eluted stepwise by buffers of increasing phosphate concentration The eluate was analysed for protein (— —) and for C6 (□ – – □) C7 (● - - - ●) and C8 (▲ ▲) activity

activities was removed in this manner After the initial washing step C 7 activity was eluted at a conductance of 11 millimhos per cm and C 6 at a conductance of 12 millimhos per cm in well separated areas The main part of the C 8 activity was eluted at a conductance of 13 millimhos per cm Traces of C 8 also occurred in the later parts of the C 6 area

Suitable fractions from the chromatogram containing one of the components C 6 C 7 or C 8 without any measurable admixture of the other two were useful as sources of C 6 C 7 and C 8 respectively Lysis of EAC 1a 4)2a 3 cells required all three of these factors as well as C 5 and C 9 Omission of any one of these five complement components from reaction mixtures containing EAC 1a 4)2a 3 cells resulted in non lytic systems

TABLE 1

Separation and Partial Purification of C6 C7 and C8

Step 1	Preparation of euglobulin	Precipitated from fresh human serum dialysed against 0 008 M EDTA of pH 5 4
Step 2	TEAE-cellulose chromatography	PO_4 buffer gradient elution (0 03 M pH 8 1 to 0 25 M NaH_2PO_4)
Step 3	Hydroxyl apatite chromatography	PO_4 buffer pH 7 9 stepwise elution (conductance 6 11 12 and 13 millimhos per cm)

It was noticed that C 7 was eluted to some extent by the starting buffer of a conductance of 8 millimhos per cm (Fig 2) Therefore in later experiments application and initial washing of the column were

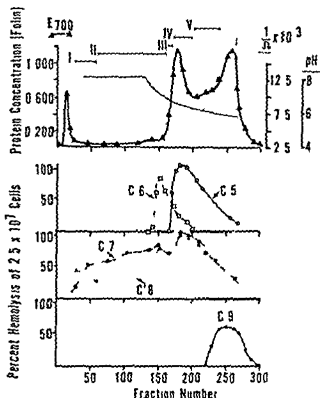


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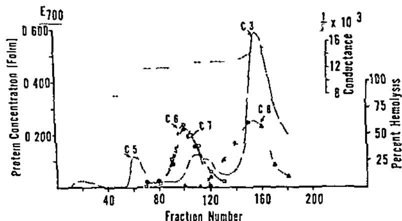


Fig 4

Chromatographic separation of C3 C5 and β_{1H} globulin on hydroxyl apatite The protein (Pool IV Fig 1) which also contained C6 C7 and C8 activity was applied in phosphate buffer pH 7 9 conductance 8 millimhos/cm The major protein constituents of the material C5 β_{1H} globulin and C3 were eluted in fractions 56–70 fractions 100–125 and in fractions 150–200 respectively as indicated by the protein curve (— —) The distribution of C6 (□ — — □) C7 (● - - ●) and C8 (▲ ▲) in the eluate was determined by haemolytic assay Fractions 56 through 70 were utilised for the further separation of C5 by preparative electrophoresis fractions 150 through 200 for the separation of C3

purified further by hydroxyl apatite chromatography (Fig 4) C6 and C7 were eluted together well separated from C3 and C5 The C8 activity largely overlapped with C3 This contamination prompted an additional purification of C3 by preparative electrophoresis As illustrated in Fig 5 C3 and C8 could be separated by this method

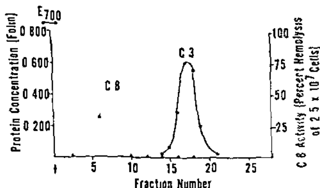


Fig 5

Separation of C3 and C8 (from fractions 150 through 200 Fig 4) by means of preparative electrophoresis in a Pevikon block A barbital buffer pH 8 6 ionic strength = 0 05 was utilized The voltage gradient was 3 5 V/cm and the separation was carried out for 21 hours Application indicated by a arrow Anode to the right C8 (▲ ▲) represented as activity C3 (●——●) as protein No protein corresponding to the distribution of the C8 activity was detected by the Folin method

C 8 migrated anodally as a fast gamma globulin completely separated from C 3 in an area where no protein was detected by the Folin method Partial overlap of C 3 and C 8 activity occasionally occurred owing to some fast migrating C 8 activity but the main part of the C 3 area was always free of C 8 activity C 8 isolated in this manner was found to become completely inactive within one to three days at +4 C when maintained in the barbital buffer

Estimation of the Degree of Purity and Physicochemical Characterization of C 6 C 7 and C 8

On a weight basis the purified fractions of C 6 C 7 and C 8 were 1100 2400 and 200 times more active than whole human serum when assayed specifically for the activity of each of the components respectively Similar estimates on a preparation of C 5 obtained according to the previously described procedure (*Nilsson & Müller Eberhard* 1965) indicated a 1400 fold increase of the specific C 5 activity

By physicochemical and immunological criteria the C 6 C 7 and C 8 preparations were heterogeneous Immunoelectrophoretic analysis of approximately 50 fold concentrates of the three preparations utilizing potent antisera against C 3 C 5 β_{1H} globulin IgA globulin and whole human serum revealed an IgG contamination of all three preparations In addition C 6 and C 8 were found to be contaminated with β_{1A} globulin and C 7 with β_{1H} globulin

Disc electrophoresis of equal amounts of the C 6 and C 7 preparations (approx 6 μg protein) in acrylamide gels and 36 μg of the C 8 preparation revealed that all three preparations were heterogeneous In addition to more slowly migrating protein two distinct protein bands were detected in the C 6 preparation (Fig 3) The faster and larger one migrated at a rate characteristic of the β_{1A} globulin The smaller one which migrated slower coincided with the C 6 active material eluted from the corresponding unstained gel C 7 activity was eluted from an area corresponding mainly to the slower one of two closely situated protein bands Protein which migrated more slowly than the C 7 active material was also visible Analysis of the C 8 preparation by acrylamide gel electrophoresis revealed multiple protein bands No haemolytic activity was recovered from any region of the corresponding unstained gel

Analysis of the distribution of the C 6 C 7 and C 8 activities upon sucrose density gradient ultracentrifugation and by Pevikon block electrophoresis in barbital buffer pH 8 6 and ionic strength 0 05 furnished some preliminary information on the physicochemical properties of the components Analysis of the purified fractions showed a sedimentation rate of 5-6 S for C 6 and C 7 and of 8-9 S for C 8 Electrophoretically C 6 and C 7 were slow β globulins The C 8 activity mi

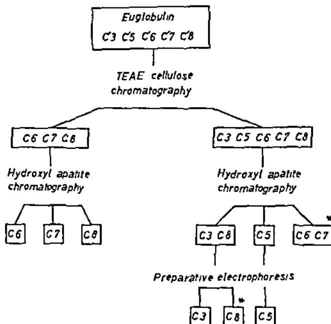


Fig 6

Outline of the separation procedure for C3 C5 C6 C7 and C8 of the human complement system Asterisks indicate discarded fractions

grated as a fast γ globulin but occasionally extended into the slow β region

Experiments with heat inactivation were performed with purified fractions of C 6 C 7 and C 8 After 30 minutes at 56 C in gelatin veronal buffer C 8 was found to be completely and C 6 partially inactivated C 7 activity remained unchanged

DISCUSSION

The procedures for the isolation of C 3 and C 5 in highly homogeneous form was described in a previous communication ([illegible] & *Muller Eberhard* 1965) In addition a factor of the complement [illegible] designated C 6 was recognized and separated from C 3 [illegible] partially purified form

The present paper deals with the further purification [illegible] the fraction possessing C 6 activity and the separation of [illegible] additional components of human complement C 7 and C 8 from this material The separation procedure which enabled the recognition of these three factors as individual components is based on the previously described method for purifying C 3 and C 5 The two initial steps of both procedures comprised the preparation from human serum of a euglobulin fraction which was further purified by TEAE-cellulose chro

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LETTERER SIWE DISEASE

A Survey and a Review of the Course in Two Subchronic Cases

By

KÅRE NYHOLM GEORGE REED and KNUD ERIK SJØLIN

Received 28 viii 66

Letterer Siwe disease has been defined as a distinct pathological entity within the group of proliferative reticuloses In 1924 *Letterer* (14) described the first case in a paper entitled Aleucamische Reticulose – ein Beitrag zu den proliferativen Erkrankungen des Reticuloendothelialen Apparates *Siwe* (28) gave an account of the clinical course and the patho anatomical changes He characterized the disease as a hyper plastic reticulosis of a non infectious nature *Abt & Denenholz* (1) named the described nosologic entity *Letterer Siwe disease*

Our knowledge of the disease originates almost exclusively from a great number of casuistic reports These reports in addition to demon strating the continual occurrence of the disease illustrate the numerous difficulties in the differential diagnosis between both benign and malignant diseases of the reticulo endothelial system Among the latter there is reason to point out 1) Infectious reticulosis 2) aleukemic monocytic leukemia 3) diffuse reticulosarcomatosis and 4) eosino philic xanthomatous granulomatoses of the types *eosinophilic granu loma of bone* and *Hand Schuller Christian disease*

Clinical and pathological experience gained from work with the latter granulomatoses led to the view of their relationship with the general reticulosis of the Letterer Siwe type This found expression in *Lichten stein s* (16) introduction of the generic term *histiocytosis X* compris ing the *eosinophilic granuloma of bone Hand Schuller Christian dise ase* and *Letterer Siwe disease* Lichtenstein based his conclusion on the transitional forms described

Though this view obtained extensive recognition a few workers have raised objections to such a simplification *Siwe* (29) stressed that the pathologist is only able to find different pathological changes within the same reticulo-endothelial system he is not therefore justified in con cluding that these indicate different stages of the same disease and cons quently a common pathogenesis *Otani* (23) supported Siwe s view

and emphasized the great difference between the clinical manifestations of the disease The eosinophilic granuloma presents itself as a localized granulomatosis running a predominantly benign course whereas Letterer Siwe disease is a diffuse reticulosis of toxic character generally running an acute or subacute lethal course

The object of the present paper is a detailed account of the symptomatology course and morbid findings in two typical cases of Letterer Siwe disease this pathological entity having been described only once by a Danish writer within the past ten years In addition we have desired to point out the continued existence of the classical general and chiefly non lipid pathological picture

REPORT OF CASES

Patient 1 (S I A Dept of Path Stanford University School of Medicine No 3F 199)

A 4 month old boy There was no familial predisposition His father was a farmer His mother a 19 year old primipara (housewife) had been extremely nauseated and vomited several times within the first trimester of pregnancy she gained about [illegible] kg during pregnancy The infant was born at term in a hospital Delivery was uncomplicated birth weight 3.9 kg The infant and mother were discharged three days after delivery

The infant was born with a dry scaly exanthem of the scalp and face At the age of two weeks he began to vomit this continued despite repeated changes in the diet. The exanthem began to spread at about the age of 2 months to cover the whole body including palms and soles of the feet two months later The skin became increasingly brittle and bled easily During the first 3½ months the infant gained 1.5 kg he then lost weight and was referred to a dermatologist Three skin biopsies led to a diagnosis of histiocytosis At 4 months of age the infant was admitted to the hospital with a diagnosis of histiocytosis X

General state on first admission—The patient was small well developed and extremely irritable Weight 5.3 kg height 6[illegible] cm B P 70/50 pulse 1[illegible]0 respiration 20 The skin was dry with a diffuse scaly erythematous exanthem most pronounced on the scalp face trunk and arms Otologic examination revealed a yellow green discharge in the left auditory canal the left tympanic membrane was not visible The right auditory canal and tympanic membrane were normal The alveolar ridges of the oral cavity were grey and brittle The lungs and heart were normal The liver was palpable 2 cm below the right costal margin and the tip of the spleen below the left costal margin There were bilateral hydrocele and a reducible right inguinal hernia The extremities presented no changes Neurological examination was unremarkable

Laboratory Tests—Hemoglobin 10.9 gm per cent hematocrit 34 per cent Leukocytes 1[illegible] 000 including 54 per cent leukocytes with segmented nuclei and [illegible] with rod shaped nuclei Microscopy of urine showed a few erythrocytes and leukocytes.

X ray Examination—The lungs demonstrated diffuse infiltrative processes which were interpreted as reticulosis of the lung tissue X ray of the skull disclosed destructive processes in the left parietal sphere and in the right orbital roof as well as questionable changes in the left occipital region The remainder of the skeletal system showed signs of destruction superiorly in the right humerus

Treatment and Course—Treatment consisted of local skin therapy The crusts were washed away with Phisohex soap In addition applications of salicylic acid sulphur precipitate ointments and Synalar were used little effect was obtained Cyt toxic therapy with Velban was instituted tentatively in intravenous doses of 1–2 mg per kilogram of body weight The treatment was discontinued after five weeks because the child s condition had deteriorated with aggravation of the skin lesions progressive enlargement of the liver and spleen progressive swelling of lymph nodes aggravation of the gingival affection loss of weight and increased irritability His condition was further complicated by acute diarrhea and con equent

dehydration which was treated with intravenous fluids including human plasma He developed an urinary tract infection by *Escherichia* and *Proteus* which was suppressed by streptomycin therapy Permanent inflammation was noticed in both ears with bilateral otorrhea from which *Escherichia* and *Pseudomonas* were cultured

Laboratory tests during stay in hospital—During the Velban treatment the thrombocyte count remained normal but the hemoglobin level fell to 7 4 gm per cent In the course of the recurrent infections the leukocyte count rose to a maximum of 25,000 and never fell below 4 000 Blood urea nitrogen remained between 13 and 15 mg per cent. Alkaline phosphatase and SGOT rose transitorily but fell again

After 53 days in the hospital the infant was discharged for continued treatment at home with Synalar ointment mandelamine methionine and vitamins as well as pHisoHex baths At the age of 6 month he was started on prednisone treatment in doses of 40 mg daily One month later the skin lesions of the face and trunk were subjected to X irradiation the total dose was 600 r The maculopapular exanthem of the entire trunk the scalp and the eyelids cleared somewhat and lymph node swelling subsided About one month later exanthem recurred on the abdominal skin followed within 48 hours by vomiting and a temperature rise to 40 5 C At the same time pronounced anemia (4 4 gm per cent) and thrombopenia (5 000) were noted The liver and spleen increased in size The child was readmitted at the age of 10 months

General state on 2nd admission—The infant was anemic and moderately indisposed B P 85/60 pulse 140 respiration 30 temperature 37 3 C Scattered petechiae were noted over the thorax A seborrheic exanthem was present behind the ears on the eyelids and scalp and in the intertriginous areas The ears could not be adequately examined because the slightest touch caused hemorrhage The lymph nodes were mildly enlarged The lungs and heart were normal The liver was palpable 4 cm below the right costal margin and the spleen was felt 3 cm below the left costal margin there was no tenderness

Laboratory tests on 2nd admission—The urine was strawcoloured and cloudy pH 5 0 no protein sugar or acetone microscopy 0-1 leukocytes 2+ uric acid crystals Reticulocytes 3 4 per cent bilirubin 0 8 mg per cent total and 0 55 mg per cent direct

Terminal course—On the second day after admission treatment with packed red blood cells was begun in doses of 10 ml/kg of body weight A total of 117 ml were transfused One of two blood cultures performed yielded growth of streptococci and the other was negative Penicillin was given The fecal guaiac test was negative except for two questionable reactions the day before death Despite transfusions the hematocrit value fell to 14 per cent on the ninth hospital day Seventy milliliters of packed red blood cells were transfused at that point the hematocrit rose briefly to 25 per cent but had already fallen to 20 per cent within 24 hours From the fifth day after admission increasing difficulty in breathing was noted together with distention of the abdomen and edema of the extremities as well as scleral jaundice The excessively low hemoglobin level raised the question of intraperitoneal hemorrhage Transfusion was resumed with fresh blood because of the thrombopenia and the infant was treated with digitalis during 24 hours because of increasing cardiac insufficiency

All treatment failed The patient s condition deteriorated rapidly with increasing dyspnea tachycardia and lethargy An over all x ray view of the abdomen disclosed increasing ascites but no distention of the intestines Electrolyte analysis the day before death showed hyponatremia The infant died one week after admission after 7 months of illness

Autopsy—Aug 13 1963

External Examination The body was that of a 17 month old boy with an anemic color of the *skin* and diffuse hemorrhagic exanthem most pronounced on the face and trunk. The exanthem consisted of massive petechiae and conglomerates of small red slightly elevated ulcerations less than 1 mm in diameter The texture and elasticity of the skin were normal The *skull* was normocephalic and the *pupils* were round and equal There was mild jaundice of the sclerae The *oral cavity* presented no changes Both *auditory canals* displayed scaly affection and purulent discharge A scaly lesion resembling seborrheic dermatitis was noted on the *scalp* and *groins* The *thorax* was symmetrical and normal The *abdomen* was protuberant and distended The *external genitalia* were normal

Internal Examination The abdominal organs were in normal positions The peritoneal lining was smooth The *peritoneal cavity* contained 200 ml of yellowish fluid There were no adhesions intestinal obstruction or dilatation The *liver edge* reached 3 cm below the right costal margin Bilateral inguinoscrotal herniae were seen

Thorax The *mediastinum* presented no changes The *thymus* weighed 6 grams was of buttery consistency and yellow Petechiae were seen on the epicardium and on both pleural surfaces most pronounced on the diaphragmatic portion of the pleura There were no adhesions and there was no fluid in the pleural cavity

Cardiovascular System The *pericardial cavity* contained a small amount of fluid Petechiae were seen in the pericardium and on the surface of the epicardium The transverse diameter of the *heart* was enlarged The heart and lungs weighed 125 grams The large vessels arose normally There was moderate coarctation of the aorta A few scattered ecchymoses were noted in the *Endocardium* The valves and ostia were normal The foramen ovale was closed Coronary arteries presented no changes

Respiratory System The *larynx trachea* and *bronchi* showed no obstructions The arterial and venous systems were normal The *lung* tissue was light red firm and contained air

Gastrointestinal System The *esophagus* was normal as was the gastric mucosa The pylorus was patent The remaining gastrointestinal tract including efferent biliary passages presented no abnormalities

Liver Gallbladder Pancreas and Spleen The *liver* weighed 1200 grams Its surface was normal Cut surface showed accentuation of the lobular pattern the color was light yellow One of the large intrahepatic bile ducts was dilated and filled with green fluid The *right hepatic duct* was firm and somewhat irregular The *gallbladder* contained clear slightly yellowish fluid somewhat dilated but the passage to the common bile duct was free The *pancreas* was firm and white and the cut surface was normal The *spleen* weighed 325 grams It was firm with a single fibrous adhesion on the surface The cut surface was dark red and homogeneous No infarctions or tumor masses were seen The mesenteric lymph nodes were greatly enlarged and mediastinal and inguinal nodes enlarged to a lesser degree The *bone marrow* was red

Urogenital System The *kidneys* weighed 75 grams each Remains of fetal lobulation were seen on the surface The capsule stripped easily The tissue was rather pale with striate marking in the medulla The calyces were somewhat dilated Pelves were normal Both *ureters* were unobstructed The *prostate seminal vesicles testes* and *epididymis* presented no gross changes

Endocrine System The *pituitary body* was not examined The *thyroid* tissue was normal No parathyroid was found The *suprarenal glands* weighed 3 gm each and were well defined The cut surface was very pale with a very slight content of lipid

Musculoskeletal System No gross changes were noted The brain was not examined

Microscopic Examination (see also Table 1)

The histological changes were characterized by an extensive and varying infiltration by large histiocytes The cells were round oblong or spindle shaped often vacuolated and about twice the size of normal macrophages The nuclei of these were often oblong rather light but with no signs of increased activity There was abundant cytoplasm A definitely foamy structure was observed only in the histiocytic cells of the thymus

In the *heart* few subendocardial extravasations of blood were seen The myocardium showed light diffuse infiltration by large mononuclear and spindle shaped cells

The *vessels* were normal

In the liver fatty vacuoles were seen chiefly periportally (Fig 1) The nuclei of the liver cells varied in size and chromatin density There was mild bile duct proliferation and the bile ducts were filled with bile The sinusoids were hyperemic focally primarily in the mid zone Many branches of the portal vein were filled with histiocytes There was compression of the portal zones and the connective tissue seemed to be compact but not increased The largest bile ducts in the right lobe were dilated and filled with bile

The red pulp of the *spleen* (Fig 2) was largely filled with or replaced by histio-

TABLE 1

Histopathological Findings

	Pt 1	*Pt 2*
n	Histiocytic infiltrates subepithe lially in the corium Local in vasion of the *epidermis A few* foam cells Focal haemorrhages	Slight focal histiocytosis in the corium Focal ulcerations in epi *thelium*
ne marrow	Narrow cartilage columns Pro nounced histiocytic infiltration of the marrow with a few foam cells and giant cells Very few eosinophilic granulocytes Ery throphagocytosis	Pronounced histiocytosis in the marrow Fibrous tissue and large foam cells in focal process in a rib
ymus	Stroma fibrous with a few calci fied foci and many dispersed *histiocytes No foam cells or* eosinophilics No Hassall s bod ies	Severe fibrosis with scattered collections of lymphocytes and *infiltration with histiocytes* Fo cal calcifications No Hassall s bodies No foam cells or eosino philics
eart	Moderate diffuse interstitial histiocytic infiltration of the myocardium No foam cells or eosinophilics	Massive infiltrates of histiocytes and foam cells in the loose con nective tissue and fat tissue around the pericardium Hydro pic degeneration of muscle No eosinophilics
ung	Diffuse histiocytosis in pleura and in adventitia of the vessels Focal histiocytos s around the terminal bronchioles No foam cells or eosinophilics	Diffuse histiocytosis in pleura and interstitial tissue No foam cells or eosinophilics
igestive ystem	Diffuse histiocytosis in the sub mucosa of the stomach small intestine appendix and colon Histiocytes in subserosal tissue of colon *Nests of histiocytes in* Peyer s plaques Foam cells ery trophagocytosis but no eosino philics Ballooming degeneration in plexus myentericus	*Osophagus scattered histiocyto* sis in mucosa Focal histiocyto sis in the appendicular submu cosa and subserosa No foam *cells or eosinophilics Diffuse* histiocytosis of mucosa and sub mucosa in the small intestine Focal histio yte infiltration in *mucosa diffuse in submucosa* and subsero a of colon Degen eration of ganglion cells
Liver	Periportal fatty infiltration with compression of the portal zones Fibrosis of periportal areas Bile duct proliferation intracapillary cholestasis *Histiocytes in the* portal venes No eosinophilics or foam cells	Mass e periportal fatty infiltra tion H stiocytosis in the peri portal spaces Many histiocytes in the portal veins No foam *ells or eosinophilics*
Spleen	Histiocytosis in the red pulp and connective tissue A few foam cells no eosinophil s Erytro phagocytosis	Histiocytosis erytrophagocyto sis giant cells a few foam cells Extramedullary haemopoiesis

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TABLE 1
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Thymus	Stroma fibrous with a few calcified foci and many dispersed histiocytes No foam cells or eosinophilics No Hassall s bodies	Severe fibrosis with scattered collections of lymphocytes and infiltration with histiocytes Focal calcifications No Hassall s bodies No foam cells or eosinophilics
Heart	Moderate diffuse interstitial histiocytic infiltration of the myocardium No foam cells or eosinophilics	Massive infiltrates of histiocytes and foam cells in the loose connective tissue and fat tissue around the pericardium Hydropic degeneration of muscle No eosinophilics
Lung	Diffuse histiocytosis in pleura and in adventitia of the vessels Focal histiocytosis around the terminal bronchioles No foam cells or eosinophilics.	Diffuse histiocytosis in pleura and interstitial tissue No foam cells or eosinophilics
Digestive system	Diffuse histiocytosis in the submucosa of the stomach small intestine appendix and colon Histiocytes in subserosal tissue of colon Nests of histiocytes in Peyer s plaques Foam cells erythrophagocytosis but no eosinophilics Ballooning degeneration in plexus myentericus	Øsophagus scattered histiocytosis in mucosa Local histiocytosis in the appendicular submu *cosa and subsero a* No foam cells or eosinophilics Diffuse histiocytosis of mucosa and submucosa in the small intestine Focal histiocyte infilt ation in mucosa diffuse in submucosa and subse osa of lon Degeneration of g ngl on cells
Li er	Periportal fatty infiltration with compression of the portal zones Fibrosis of periportal areas Bile duct proliferation intracapillary cholestasis Hist ocytes in the portal venes No eosinophilics or foam cells	Massi per rt l fatty infiltration H tio t is in the periport l p e Many histiocytes in the portal veins No foam ells sinophilics
Spleen	Histioc tosis in the red pulp and connective tissue A few foam cells no eosinophilics. Erytrophagocytosis	H tio ytosis erytrophagocyto s giant cells a few foam cell Ext amedullary *haemopoiesis*

TABLE 1 (cont.)
Histopathological Findings

	Pt 1	Pt 2
Pancreas	Histiocyte infiltration in the interstitial tissue. No foam cells or eosinophilics	Many histiocytes in the capsule and interstitial tissue. In the tail an accessory spleen with histiocytes. No foam cells or eosinophilics
Kidney	Numerous histiocytes in the small veins and lymph vessels. A focus of histiocytes in the interstitial tissue. No foam cells or eosinophilics	Fresh infarct in the cortex. Focal histiocytosis in the perivesicle fat and capsule
Urinary bladder		Moderate focal subepithelial accumulation of histiocytes.
Suprarenal gland	Histiocytosis in the reticular zone and round the veins in the medulla. Few foam cells. Histiocytes in the surrounding fat	Local histiocytosis in the capsule
Parathyroid		Diffuse infiltration of histiocytes in interstitial tissue and capsule. No foam cells or eosinophilics
Thyroid		Diffuse infiltration of histiocytes in capsule and connective tissue. No foam cells or eosinophilics
Hypophysis		Diffuse histiocytosis with foam cells in posterior lobe. Granuloma of foam cells in adenohypophysis. No eosinophilics
Lymphnodes	Diffuse histiocytosis in sinus and capsule. No foam cells or eosinophilics	Diffuse histiocytosis in sinus. No foam cells or eosinophilics
Central nervous system	The fat surrounding cauda equina contains infiltrates of histiocytes with many foam cells. No eosinophilics	Histiocytes in the adventitia of the meningeal vessels
Middle ear		Connective tissue massively infiltrated by histiocytes and many foam cells. No eosinophilics

cytes. The histiocytes varied in size. Some contained rather abundant cytoplasm with clear vacuoles while others contained fragments of nuclei and a small number of phagocytized erythrocytes. Very few had foamy cytoplasm. The white pulp and the lymphatic follicles were not conspicuous. No hemorrhages or necroses were seen. The *esophagus* presented no infiltration by histiocytes. Below the level of the esophagogastric junction histiocytes were seen scattered through the submucosal layers of the *stomach*, *small intestine*, *gallbladder* and *appendix*. *Peyer's plaques* were



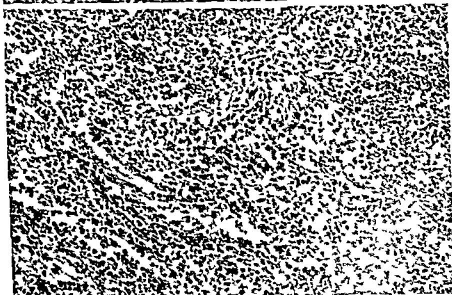


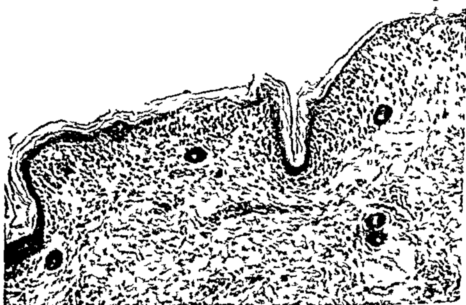
Fig 1 Fatty degeneration of the li er cells especiall in th | [t l area
Fig 2 The Spleen Irregular infilt ates with histiocyte in th red p lp is observed

filled with nests of hist o ytes and lymphocytes Perit n l ling with accumulation of abnormal histiocytes was seen focally

In most *sk in* areas (Figs 3 and 4) the basal laye f th epidermis was intact In some places the histioc tes invaded the middle la r the epidermis but not the granular and horny layers Focal intradermal hem r hage were seen imm diately below the ba al membrane

Peripheral cells in the glomeruli of the *kidney* cont ned hyperchromatic nuclei





Figs 3–4
The skin The subepidermal part of corium is massively infiltrated with histiocytes The infiltrate is continuous and band like

The tubules were normal with mildly vacuolated cells Numerous histio ytes were seen in the thin walled veins and lymphatic vessels The interstitial tissue and arte ries were normal

The *pleurae* were thickened and contained a large number of histiocytes scattered partly loosely along the interlobar fissures partly in the arterial and venous ad





Fig 5 The bone marrow Most of the *myeloid tissue is repl ed l h* stiocytes
Fig 6 Fibrous adhesion from the right pleura The tissue is h a ily infiltrated with histiocytes a d of few lymphocytes and plasmacell Th l olar septa are similar thickened

ventitia Similar cells were al o found in focal a r ng ment around the terminal bronchioles In the *suprarenals* histiocytes were pre nt n the reticular zone and around the medullary veins No foam c lls were s en The *testes* were immature and trophic The fibrous stroma of the *pancreas* conta n d a number of histiocytes. The acini and insular tissue were normal The fatty and onnective ti sue of the *thymus*

was diffusely infiltrated by histiocytes. No lymphatic tissue was left. Calcified foci were seen encircled by fibrous rings. The latter were surrounded by *mononuclear* cells. No normal Hassall's corpuscles were left. The columns of cartilagenous tissue in the *bone marrow* were abnormally narrow. The spongiosa trabecles in the bone marrow cavity were normal. The marrow (Fig. 5) was infiltrated by histiocytes and foci of erythropoid and myeloid tissue as well as megakaryocytes were seen in some places. The spinal cord was normal.

Patient 9 (R.R. Dept. of Pathology Stanford University School of Medicine No. SF 19)

A 17 month old girl. No similar diseases were known in the family.

Pregnancy and delivery were uncomplicated, birth weight 2.6 kg. The infant was sound and developed normally in the first six months of life. She was able to sit at the age of 5 months, to crawl at 7 months, and to walk at 11 months.

Her weight increased normally during the first year of life.

When the infant was 6 months old, the mother noted a napkin area dermatitis. At the same time, an exanthem occurred on the scalp. This did not respond to ordinary skin therapy. Both lesions were aggravated at the age of 8 months and continued to be resistant to various forms of local therapy.

At the age of 12 months, the infant was admitted for a short time to another hospital, where a diagnosis of histiocytosis X was made on the basis of skin biopsies from the back and genital region. At the same time, liver and spleen enlargement were diagnosed. X-ray of the thorax revealed a destructive lesion in the left 5th rib. Hemoglobin 10 gm per cent, leukocytes 10,000/cmm.

Steroid therapy was instituted and the affected skin areas were submitted to superficial X irradiation. During the next two months, Kenalog was given in doses of 16 mg daily; this was then replaced by Aristocort. The treatment was continued until her admission to the Stanford University Medical Center three months later. During the same period, different skin areas were irradiated by a total of 1690 r. At the age of 14 months, her development had regressed; she ceased crawling and doing other things learned previously. Her appetite was declining and there was intermittent rise of temperature. Ulceration of the extremities, chronic otorrhea and polypnea were also noted.

She was admitted to Stanford Medical Center at 17 months of age.

General state on 2nd admission.—The infant was malnourished. Pulse 130. B.P. 90/64, respiration 64. The scalp exhibited scaly exanthem with bleeding areas. The skin of the back displayed a dry, scaly exanthem and of the buttocks an erythematous, moist exanthem. Bilateral lymph node swelling was observed on the neck; individual nodes were enlarged up to 3 cm in diameter. There was no axillary node swelling. A greenish discharge was noted in both auditory canals. The patient's face was Cushingoid. The eyes were normal. The teeth were in extremely bad condition. The thorax was normal. The liver was palpable 7 cm below the right costal margin. The spleen was firm, extending to 4 cm below the left costal margin. The back and extremities presented no changes.

Laboratory tests. Urinalysis: pH 6.0, specific gravity 1.018, microscopy 1-2 erythrocytes and 4-6 leukocytes per visual field. No sugar or protein. Hemoglobin 6.7 gm per cent. Leukocytes 4,000-6,000/µ. Differential: Rod shaped leukocytes 2, segmented leukocytes 40, lymphocytes 49, monocytes 8. Serum electrolytes normal. Bilirubin 16 mg per cent direct, 1.2 mg per cent total. Prothrombin time over 100 per cent. Bleeding time 6.5 min. Serum protein 5.6 gm per cent, Albumin 2 gm per cent, globulin 3.6 gm per cent. Bone marrow aspirate: Histiocytosis and mild erythroid hyperplasia. X-ray of the bony system showed a destructive process in the left fifth rib. No other changes were noted in the skeletal system.

Course during stay in hospital.—Of several blood cultures, none showed growth of bacteria. A urine sample drawn by suprapubic puncture was sterile. Coagulase negative staphylococci were cultured from the skin and ears. Excessive growth of *Pseudomonas* and *Proteus* was demonstrated in the auditory canals. The treatment consisted of antibiotics for the skin and ear infections as well as *local skin therapy* in the form of Synalar shampoo and ointment and Cetar shampoo. Burow's solution was used for drying of the napkin area dermatitis. Penicillin and Streptomycin did not alter the course.

After one week in the hospital, the hemoglobin level fell to 4.9 gm per cent. Trans

fusion of 100 ml of packed red blood cells treatment for five days with prednisone in doses of 100 mg daily continued X irradiation of the affected skin areas and treatment with Cytotoxan and Velban had no influence on the progressive course The condition deteriorated two weeks after admission associated with tachycardia tachypnea and increasing ascites. Death occurred 24 hours later during blood transfusion and after 13 months of illness Artificial respiration and cardiac massage were of no avail

AUTOPSY

External Examination

The body was that of a 17 month old girl Weight 7 5 kg length 70 cm The *scalp* exhibited ulcerations and cicatrization particularly pronounced along the hair line Macerated ulcerations were seen on the skin covering the *trunk* The ulcers measured up to 0 5 cm in diameter Ulcerated areas were red and alternated with scattered petechiae which were particularly conspicuous over the upper part of the thorax and lower part of the abdomen The *vulva* was edematous The *skull* was normocephalic The *pupils* were round and equal. The sclerae were not jaundiced. A greenish mucous discharge was found in both auditory canals and in the oral cavity The postauricular and submandibular regions were moderately edematous The *thorax* and the *abdomen* were of normal shape The *liver* was palpable 3–4 fingerbreadths below the costal margin The *spleen* was palpable The *extremities* presented no gross changes

Internal Examination

The Thorax the mediastinum was fatty The *thymus* weighed 6–8 grams The tissue was firm and fibrous with streaks of yellow buttery tissue The *mediastinal lymph nodes* were not enlarged The *pleurae* were smooth The *pleural cavity* contained a very small amount of serous fluid A firm fibrous adhesion measuring 1×? cm extended from the lower part of the left upper lobe to the costovertebral angle

Cardiovascular system The *pericardium* contained 18 ml of yellow vis ous fluid The 56 gram *heart* was not enlarged The large vessels arose normally The valves were normal The foramen ovale was closed The wall was of normal thickness The *endocardium* presented no changes There was no fibrosis or fresh hemorrhage in the wall The *coronary vessels* were normal Small yellow white intimal plaques were seen in the *aorta* The arch was normal At the descending aorta, a fibrous band 10×? mm was seen at the site of the closed ductus Botalli None of the large intrathoracic or abdominal arteries or veins exhibited any changes

Respiratory system The *larynx trachea* and *bronchial tree* were grossly normal except for hyperemia of the mucous membrane An abundant mucous discharge was found in the fairly small bronchial branches There was no definite obstruction The arterial and venous systems presented no changes Lung tissue was only m derately crepitant its color was light red apically and darker red ba ally On ut section a copiou amount of foamy fluid was expressible Around the left pleural adhesion no changes were found within th lung tissue The hilar node were n t enla ged

Gastrointestinal system The abdominal organs were situated normall Th peritoneum c ntained 100 ml of sl ghtly cloudy fluid There were no he e or intestinal obstructions The pylorus was patent

The *liver weighe l 7?o grams The cut surface presented a la* [illegible] te l m rking apparentl indicative of portal fatty infiltration The t c a pale and brownish The *gallblad ler* was slightly dilated and c nt n d 10 m of gr en bile The mucosa had yellow streaks The *common bile duct* was p te t Th *intrahepat c bile duct system* was normal The *pancreas* was norm l A n ll ur ber f small yellow spots were seen on the surface of the serous m mbr n f t surface was normally lobulated The *spleen* weighed 1 ? grams It w f m and purple The cut surface displayed cattered small g ey foci The *abdominal ly ph n des* were not enlarged The cut surface was normal The *bone ma w* w s r d Small foci of white marrow were een especially in the lower p rt f the tel ral column These w re presumably infiltrates of abnormal cells In the *left f th rib* a fluctuating semi flu d yellowish mass was found to which th lung adhered In this area there was osseous destruction and extension of the d stru tive pr cess into the vertebral b dy and further around the spin l canal

Urogenital system The *kidneys* weighed 32 grams each Residual fetal lobulation was seen on the surface The color was pale red and cut surface showed the medulla to be somewhat lighter than the cortex The calyces and pelves were not dilated The *ureters* were free and the bladder slightly dilated The vesical mucosa presented focal hemorrhages No changes were seen in the internal genitalia

Endocrine system The *pituitary body* was firm It was 1 cm in diameter The *thyroid parathyroid* and *suprarenal glands* presented no changes The cut surface of the suprarenal glands showed the cortex to be pale yellow

Musculoskeletal system The muscular tissue was firm and red It was mildly edematous in the upper part of the body Osteolytic changes were demonstrated in the anterior middle and posterior cranial fossae and in the calvaria these being filled with a soft yellow material

Central nervous system The brain weighed 850 grams The gyri were not flattened The meninges were clear and the vessels normal The left eye was removed and submitted to microscope which revealed no abnormality Part of the *spinal cord* was examined and found to present no morbid changes The meninges covering the petrous portion of the temporal bone contained nodules of a firm buttery tissue measuring 0 75 cm in thickness Numerous coronal sections through the cerebrum showed the cortices thalamus mesencephalon pons medulla oblongata and cervical portion of the spinal cord to be normal with no abscesses ecchymoses or other abnormalities *There were no intraventricular ecchymoses or petecchiae*

Microscopic Examination

Heart The endocardium and myocardium were normal A coronary artery in the left ventricle exhibited moderate eccentric focal intimal thickening A focus of intimal thickening was seen in the aorta This presented atheromatous vacuolation Histiocytic infiltrates were found in the loose tissue around the pericardium

Lungs and pleurae The pleurae were thickened edematous and contained histiocytes The alveoli contained lymphocytes macrophages and some residual cell elements Scattered accumulations of histiocytes were found in the parenchyma some distance from the pleural surface An increased number of lymphocytes and a small number of leukocytes were demonstrated in the alveolar septa The bronchi and vessels were normal (Fig 6)

Massive periportal fatty infiltration was found in the *liver* cells There was no necrosis of the liver cells A few nuclei were vacuolated The portal spaces contained a moderate number of abnormal histiocytes In the central areas the liver cells were of normal appearance Dilated sinusoids were found to contain leukocytes Kupffer s cells and abnormal histiocytes The walls of the central veins were somewhat thickened A few normoblasts were seen In the *spleen* the sinusoids of the pulp were dilated and contained numerous hemosiderin filled macrophages Active hematopoiesis was demonstrated In between histiocytic elements were seen In some areas they contained vacuoles and in others phagocytized erythrocytes Many of the nuclei were notched others were multilobular and had no peculiar mitotic figures Here and there were nests consisting of 6 8 cells with round moderately basophilic and vacuolated nuclei The cytoplasm was pale blue and well defined The cells were oblong spindle shaped and 2–3 times the mean size of normal macrophages The capsule was fibrous but not thickened The arterioles presented no changes

The *gallbladder* was normal In the tail of the *pancreas* was an accessory spleen containing infiltrates of atypical histiocytes The acini and islets of Langerhans were normal The connective tissue around the pancreatic duct and the vessels was increased in breadth and contained a fairly large number of histiocytes

Gastrointestinal canal The esophageal submucosa showed lymphocytic infiltrates the mucous membrane was normal The stomach small intestine and colon displayed no microscopic changes The lymph follicles of the appendix were replaced by focal collections of histiocytes

Urogenital system The glomerular coils of the kidneys were well defined and the tubules well preserved The interstitial and vascular tissue were normal where s histiocytes were observed in the peripelvic fat

Endocrine system Moderate accumulation of lipid was seen in the cortex of the suprarenal glands The reticular zone was fairly rich in cells The medulla presented no changes Histiocytic infiltrates were observed in the peri glandular fatty tissue (Fig 7)

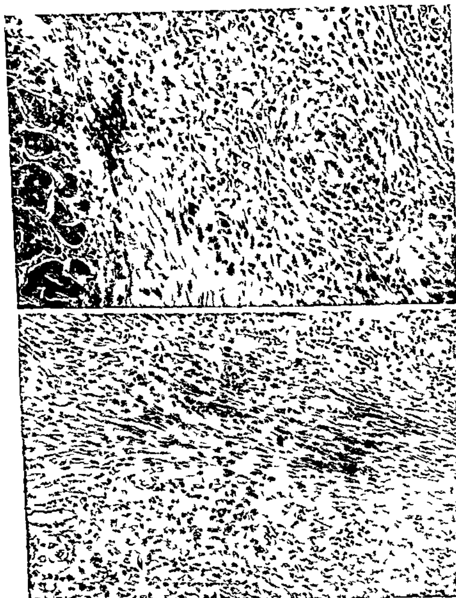


Fig 7 Capsule tissue of the adrenal Zona glomerulosa is seen to the left. The capsule and the surrounding fat tissue is infilt ated with a multitude of histiocytes and a few lymphocytes

Fig 8 Tumour like tissue from the middle ear It consists of onnective tissue with massive infiltration of histiocytes many lymphocytes and a few plasma cells

The posterior lobe of the pituitary body was partially replaced by histiocytes The anterior lobe was normal The thyro d was without changes The parathyroid con tained histiocytes in the interstitial tissue The *ovary* contained numerous oocytes No Hassall s corpuscles were detectable in the *thymus* but scattered lymphocytic

infiltrations were seen with moderate fibrosis and diffuse infiltration by histiocytes, as well as local areas with calcification possibly remnants of Hassall's corpuscles.

In the *skin* the epidermis exhibited focal ulceration with basophilic debris and infiltrations of leukocytes and lymphocytes in the necrotic areas There was scant focal infiltration by histiocytic cells in the corium

Central nervous system An increased amount of fibrous tissue was found in the meninges about the petrous portion containing histiocytic infiltrates Under polarized light the histiocytes were seen to contain anisotropic substance which gave a positive reaction to fat staining The spinal cord (cervical and lumbar portions) the mesencephalon and the medulla oblongata were normal Purkinje's cells in the cerebellum were intact In the pons a few of the neurons were globoid but showed no other definite signs of degeneration The frontal parietal and occipital cortices and the thalamus were normal

The bone marrow contained a great number of histiocytes with an amorphous eosinophilic cytoplasm The marrow of the skull was replaced by histiocytes *Tumor* like tissue from the middle ear consisted of connective tissue massively infiltrated by histiocytes and several lymphocytes and plasma cells (Fig 8) At the site of the radiographically demonstrated process in the left fifth rib the marrow was infiltrated by fibrous tissue large foam cells and large cells with basophilic cytoplasm and small pyknotic nuclei The hematopoietic tissue was partially replaced. A small number of megakaryocytes was found

DISCUSSION

The two cases reported illustrate the subchronic form of Letterer Siwe disease The earliest publications (1) considered only the acute form running a fatal course within a few weeks or months

At Hospital for Sick Children Great Ormond Street (6) 16 cases of reticulo-endotheliosis were diagnosed within a period of 30 years Among these were only six disseminated forms (the Letterer Siwe type) Within the same period 18 lymphosarcomas 17 malignant lymphogranulomatoses and 91 neuroblastomas were treated at that hospital

In Denmark 19 cases of Letterer Siwe disease have been diagnosed within the past 20 years among which were five atypical forms (20) A few reports from American hospitals (11 19 21) give a similar incidence pointing out at the same time the diffuse chiefly non lipid reticulosis as the rarest form of the trilogy termed histiocytosis X

The earliest characterization of the symptomatology was based on knowledge of the acute forms where the disease was generalized at the time of first admission (1) The subchronic form has been described in subsequent reports (3 25 26)

The interest displayed in the initial symptomatology centers particularly on factors contributing towards an earlier diagnosis in the hope of thereby obtaining better results of treatment Schäfer (27) pointed out the initial occurrence of the skin lesions and subsequent publications (19 21 25) largely bore out this observation In the two patients described above dermatosis of a seborrheic character was the initial sign In the former this sign was present at birth In both cases the seborrheic lesions were resistant to ordinary skin therapy whereas it abated in response to subsequent X irradiation In the former patient the lesion was complicated by ulcerations and hemorrhage In both patients the diagnosis was confirmed by skin biopsy (Figs 3 and 4) This

initial monosymptomatic phase of L S disease is often of several months duration (20 24) Reports in the literature show that the disease is often not diagnosed until it has reached its generalized form at which stage all therapy has been futile thus far Better results of treatment therefore cannot be obtained unless the disease can be diagnosed at an earlier stage Accordingly diagnostic skin biopsy is advisable in cases where a lesion of the type of seborrheic dermatitis in infants between 0 and 3 years of age proves resistant to treatment

Histiocytic and reticular cell infiltrates occur superficially in the corium in lymphogranulomatosis mycosis fungoides juvenile xanthogranuloma and H S C disease among others A differentiation from the two former types of disease is hardly difficult in practice Letterer Siwe disease occurring almost exclusively in the 0– to 3 year age group in which age class the 2 firstnamed diseases are extremely rare

Whereas dermatosis are almost constantly present in Letterer Siwe disease and generally from the outset they occur according to *Paulson et al* (24) in no more than one third of patients with Hand Schuller Christian disease where they are most often secondary to other signs and symptoms (20)

The clinical appearance and histological picture of the skin lesion is about the same in the two diseases though the more chronic forms of Hand Schuller Christian disease may present xanthoma cells in the reticular infiltrates in the corium (24)

The progression of the disease is characterized by bilateral otorrhoea and cervical and more general lymph node swelling Processes of bone destruction are revealed by x ray examination as sharply delineated rarefactions The appearance is not distinguishable from the osseous processes in eosinophilic granuloma and Hand Schuller Christian disease but unlike the bone lesions in these two diseases localization in the bony system in Letterer Siwe disease is most often an asymptomatic radiographic finding In the above two diseases the osseous processes will give early clinical signs and symptoms owing to the rapid growth This causes dilatation of the medullary cavity and penetration through the cortical substance in some cases resulting in spontaneous fracture A universally demonstrated affection of the bone system is rare in L S disease radiographically A more frequent finding is an asymptomatic solitary cyst (as in Patient 2) but in many cases radiographic rarefactions are totally absent in the skeletal system (22) Most of the postmortem findings reported have revealed a generalized but not particularly intense reticulosis in the bone marrow as opposed to a more localized and excessively proliferating granuloma formation in eosinophilic granuloma and Hand Schuller Christian disease These findings also lend support in favor of a difference with regard to the osseous manifestations between the general chiefly non lipid and the focal xanthomatous form of histiocytosis

Liver and spleen enlargement are cardinal signs which in the more

protracted forms occur at a late stage (26) They indicate generalization of the disease and are consequently an unfavorable prognostic sign

The most common laboratory tests are of little aid in the diagnosis The disease is associated with progressive anemia blood transfusion has had no more than transitory effect Whether this is caused by a hemolytic factor is so far unknown *Tipton* (17) described a hemolytic and pancytopenic phase of the disease but hemolysins have not been demonstrated thus far (11 20) However erythrophagocytosis is a common phenomenon in the autopsy material Terminal jaundice could be explained by the pathological processes involving the liver resulting in compression of the portal spaces (Patient 2) An elevated alkaline phosphatase is found in Patient 2 bears out the obstructive character of the icteric state

The findings of pigment deposits in the proliferated reticular cells in connection with areas of extramedullary hemopoiesis bear great resemblance to the pathologic findings in hemolytic anemia They may however be explained by the presence of hemorrhages in the tissues together with displacement of the erythropoid tissue in the bone marrow

Moderate leukocytosis is often an initial finding (7 27) The rise of the total leukocyte count has been attributed to complicating infections In our case 2 the latter were accompanied by peripheral leukocytosis which gradually changed into mild leukopenia The frequent leukocytosis in the initial phase has given rise to the question whether the infectious component plays a part as a factor releasing and accelerating the abnormal pathological growth The terminal phase is marked by a normal leukocyte count or mild to moderate leukopenia (1) even in the presence of infection Depressed leukopoiesis in this phase may account for the absence of leukocytosis

Careful bacteriological examinations have revealed numerous complicating infections in infants with Letterer Siwe disease but it has thus far been impossible to establish their definite responsibility for the extensive pathological changes Bacteriological examination in the *present cases revealed the presence of different organisms in the* skin (Staphylococcus) and ear tissue (Pseudomonas Proteus) In both infants postmortem examination showed bacteria in different organs Further previous reports on fatal septicemia (3 5 8 10 26) add emphasis to the hazards of this complication especially in cases submitted to steroid therapy A few descriptions of a non lethal course after antibiotic treatment (2 4 12) must give added interest in the problem of the significance of these bacterial infections possibly as part of a more complex genesis

Our two patients were both submitted to steroid treatment We can not say whether this treatment was responsible for the protracted course Lasting improvement was not obtainable however not even after administration of an excessively large steroid dose (100 mg prednisone daily to patient 2)

Thus a greater interest should be taken in a combined treatment comprising a large steroid dose and a broad antibiotic therapy A few reports have been published of favorable responses to this (13 15)

Cytotoxic therapy has been ineffective so far having even in some instances aggravated the clinical state or caused leukopenia and thrombopenia (13 26 30) These results are in accord with the apparent exacerbation following Velban therapy to our patient X irradiation of the skin lesions osseous foci and abdomen has given palliative effect but of only short duration (26) In patient 1 this therapy caused remission of both the exanthem and the lymph node swelling

Thus the treatment of L S disease has given only discouraging results The disease must still be regarded as almost 100 per cent lethal Nevertheless a few reports of non lethal courses together with more and more frequent observation of a protracted course after intensified therapy must stimulate continued therapeutic investigation

In the preceding discussion attention has been called particularly to the demand for an adequate therapy with antibiotics in association with steroid therapy The necessity of such treatment is evident from the results of the bacteriologic examinations in the two patients described above

The pathological changes are characterized by proliferation of atypical reticular cells in different organs The proliferating cells displace the normal cells thereby producing the characteristic changes in various organs In the skin the infiltrates occur subepidermally with local invasion and ulceration of the epidermis (24) In the bone marrow the hematopoietic tissue is displaced being often seen as islands of active erythroid and myeloid tissue surrounded by dense tracts of histiocytes In the lymph nodes and the spleen the follicular system becomes atrophic while the sinus and the medulla are subject to varying degrees of reticulosis In the liver hepatic cells undergo fatty degeneration which is periportal in its primary phase (patients 1 and 2) Infiltrates of histiocytes and an increased amount of connective tissue are often seen in portal spaces Histiocytic cells may be seen in the sinusoids and in the central veins (patient 2) Scattered histiocytes are often observed in the thymus where however fibrosis may predominate associated with degeneration or total atrophy of Hassall s corpuscles Atypical histiocytic cells may be found in any organs but are here often scattered along the vessels and in the connective tissue

The pathological histiocytic cell has been described by numerous writers (1 28) It may vary in shape size and appearance but there is almost general agreement about the non malignant character of the cell The cells may contain small vacuoles but proper xanthomatosis with foam cells containing doubly refractive substance (patient 2) is a rare finding and if present a local phenomenon (20) The cells occur either in well defined infiltrates or in the form of a diffuse infiltration The changes may be accompanied by presence of giant cells of

atypical appearance, extramedullary hemopoiesis, necrosis and hemorrhages.

The degree of reticular cell phagocytosis is high, a fact which often characterizes the picture and which caused *Farquar & Claireux* (9) (1952) to employ the term "hemophagocytic reticulosis".

Otani (23) stressed the highly toxic disease picture and suggested a viral etiology as the cause of the general changes. *Siwe* (29) pointed out the resemblance of some of the published cases to leukemia and similar diseases. He suggested an autoimmune mechanism as a possible etiological explanation. *Herman & Dargeon* (18) pronounced that the classical Letterer-Siwe disease resembles much more a generalized reticular cell sarcoma or monocytic leukemia than a granulomatous disease, but this opinion has not been supported by subsequent writers.

The views regarding the etiology thus differ considerably. This is due in part to certain variations in degree of the histopathological changes which in some instances tend toward a malignant picture. This variation *may suggest a difference in etiology, but may just as well be regarded* as evidence in favor of a difference in toxicity. Specific toxins have not been demonstrable so far, but the variation in the nature of the bacterial infections seems to indicate that these are secondary, although we cannot exclude the possibility that they may activate and accelerate a latent disorder in the RES.

In the two cases presented here, the pathological findings corresponded with the classical picture of the universal changes. Further they accentuated the general attack of the disease on the reticulo-endothelial system.

SUMMARY

The course, results of treatment and postmortem findings in two typical cases of *Letterer-Siwe disease* are reported for the purpose of illustrating the subchronic form of the disease.

The difficulties with regard to differential diagnosis are discussed briefly, with special reference to the relation of the disease to the chronic granulomatosis of the *Hand-Schuller-Christian* type and the eosinophilic granuloma of bone.

The small influence of therapy on the course of the disease and the risk of fatal septicemia, especially during steroid treatment, are demonstrated in the two cases presented and are borne out by cases described in the literature. The conclusion is drawn that the best results may be obtained by combining a large steroid dose with broad antibiotic treatment.

The pathological findings are described in detail, and the differences from the xanthomatous picture in Hand-Schuller-Christian disease with the eosinophilic granuloma of bone are pointed out.

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STRUCTURE OF THE JUXTAGLOMERULAR APPARATUS IN KIDNEYS SITUATED CENTRALLY AND PERIPHERALLY TO AN EXPERIMENTAL COARCTATION OF THE AORTA IN DOGS

By

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Coarctation of the aorta is a congenital narrowing of the aorta localized in typical cases at the ligamentum arteriosum If the narrowing is pronounced it gives rise to haemodynamic changes Hypertension and a high pulse pressure central to the narrowing and a faint or impalpable pulse peripheral to it are pathognomonic of the anomaly (*Hudson* 1965)

As to the pathogenesis of the hypertension three hypotheses prevail (*Edwards et al* 1965 *Hudson* 1965)

1 The renal hypothesis The hypertension is supposed to be due to an increased release of vasopressor substances or a reduced release of antipressor substances from the kidneys because of the haemodynamic changes caused by the coarctation

2 The mechanical hypothesis The hypertension is supposed to be due to the increased resistance which the heart has to overcome in order to pump the blood through the narrowed aorta and collaterals

3 The vascular spasm hypothesis The altered pulse pressure in coarctation of the aorta is supposed to cause an increased peripheral resistance with consequent elevation of the blood pressure

In an effort to elucidate these problems we studied the histological changes of the kidneys in dogs with experimental coarctation In particular we were interested in ascertaining whether given haemodynamic alterations caused specific and uniform changes in the juxtaglomerular apparatus of the kidneys For a detailed description of the method and results the reader is referred to a publication by one of the authors (*Svane* 1968)

Aided by grants from *Statens almindelige Videnskabsfond* and *P Carl Petersens Fond*

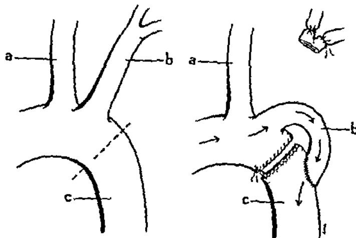


Fig 1

Method for inducing experimental coarctation of the aorta a modification of *Blalock & Park s* experimental by pass operation a brachiocephalic artery b left subclavian artery c descending aorta

MATERIAL AND METHODS

Young mongrel dogs were subjected to operation establishing coarctation of the aorta autotransplantation of the kidney to the neck and nephrectomy in different sequence and at different intervals In this way the kidneys were exposed to different but controlled haemodynamic conditions whereupon they were removed and studied histologically

Measurement of the blood pressure was done by direct puncture of the carotid and femoral arteries under general anaesthesia with 10 mg Nembutal sodium and 12 mg Leopental (pentothal sodium) per kg body weight injected intraperitoneally The manometer was an inductive transducer with an electronic amplifier A mingograph was employed as writer The mean blood pressure is the geometrical mean pressure estimated on the traced curves or measured direct using electrical damping of the pressure curves

Coarctation of the aorta was induced by a modification of *Blalock & Park s* (1944) experimental by pass operation for coarctation of the aorta (Fig 1) The left subclavian artery was divided high in the chest the distal lumen was ligated while the proximal lumen was anastomosed end to side to the aorta which was thereafter divided between the departure of the left subclavian artery and the anastomosis.

Transplantation of the kidney was carried out by a technique whose principle is identical with that described by *Pierce & Varco* (1964) The kidney was removed through a conventional lumbar incision and placed in a pocket beneath the sub cutaneous muscle on the lateral side of the neck and the renal artery and renal vein were anastomosed end to-end with the common carotid and the external jugular vein respectively The ureter was passed through a small incision in the skin more medially on the neck forming a cutaneous ureterostomy

Histological study The kidneys were cut immediately after removal and a slice of renal tissue a few mm thick was fixed in Helly s solution for 4–6 hours while the remainder of the kidney was fixed in 4 per cent neutral formaldehyde After fixation the tissue was dehydrated embedded in paraffin and cut in the usual way The sections were stained with haematoxylin-eosin periodic acid Schiff haematoxylin (PAS staining by the method of MacManus) by the methods of van Gieson Hansen and Bowie (*Bowie* 1935 *Bing & Kazimiere ak* 1964)

The juxtaglomerular apparatus was studied in PAS stained and Bowie stained sections The juxtaglomerular rating (*Schmid & Graham* 1962) used has been found to be better suited for the histological assessment of the renin content of the dog kidney (*Schmid & Graham* 1962 *Takats et al* 1962) than the calculation of the

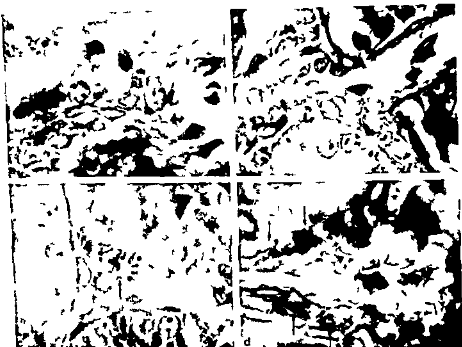


Fig 2

The juxtaglomerular apparatus in juxtaglomerular rating plus 1 (a) plus 2 (b) plus 3 (c) and plus 4 (d) Bowie × 1200

granulation index used by *Hartroft & Hartroft* (1953) on rat and mouse kidneys This rating is as follows

- Plus 1 Normal looking vascular pole with predominantly smooth muscle cells in the arteriolar walls without hyperplasia or increased stainability (Fig 2a)
- Plus 2 Enlarged vascular pole with increased stainability and with h pe pl ia of the cells in the arteriolar media but without cytoplasmic granul s (Fig 2b)
- Plus 3 In addition to hyperplasia of the medial cells dist nct c to lasm g nules in one or two sites in the juxtaglomerular apparatus (Fig 2)
- Plus 4 Numerous granules in several s tes of the juxtaglomerula rpar tus (Fig 2d)
- Plus 5 Granules showing massive spread or aggregates of—at n e t mate—half the cells in the juxtaglomerular apparatus

RESULTS

In accordance with the sequence of the procedures induction of coarctation transplantation of the kidney and nephrectomy the dogs were divided into 4 groups (Fig 3) The typical alterations in the blood pressure are shown in Figs 4 5 6 and 7 which give the blood pressure findings in dogs T 16 22 26 and 30 representing group 1 2 3 and 4 respectively It is outside the scope of the present paper to describe the details of the blood pressure changes but in outline it may be stated

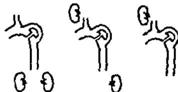








Fig 3
Grouping of the dogs by the sequence of performing the coarctation the renal transplantation and the nephrectomy

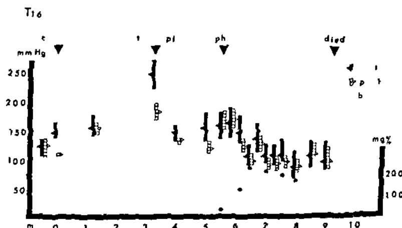


Fig 4
Blood pressure findings in a dog T 16 representing group 1 (Fig 3) The hypertension appearing after the induction of the coarctation disappeared when one kidney was transplanted to the neck and the other kidney removed

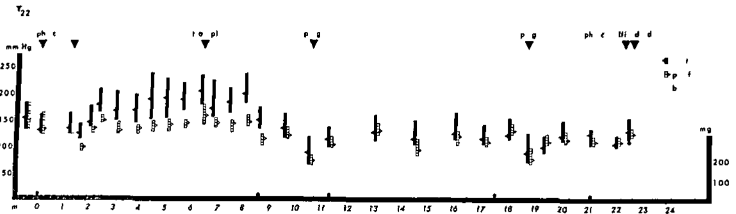


Fig 5

Blood pressure findings in a dog T 22 representing group 2 (Fig 3) After removal of one kidney and induction of coarctation the blood pressure increased but decreased again when the other kidney was transplanted to the neck

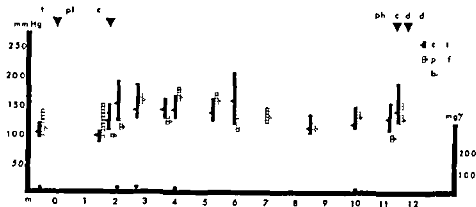


Fig 6

Blood pressure findings in a dog T [illegible] representing group 3 (Fig 3) After trans plantation of one kidney to the neck and induction of coarctation the blood pressure increased The dog died of uraemia after removal of the abdominal kidney

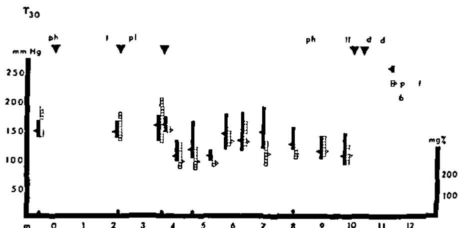


Fig 7

Blood pressure findings in a dog T 30 representing group 4 (Fig 3) After removal of one kidney and transplantation of the other kidney to the neck induction of coarctation did *not* cause any increase in the blood pressure

1 The blood pressure rises when coarctation is induced and one or both kidneys are placed peripheral to the coarctation

2 This elevation of the blood pressure does not disappear when one kidneys is transplanted to the neck (central to the coarctation) not un til the other kidney is removed

3 No elevation of the blood pressure occurs when neither kidney is peripheral to the coarctation at the time that it is induced

A juxtaglomerular rating was done on 18 kidneys removed from dogs of the 4 groups mentioned at various intervals after the surgical proce

Fig 8

Relation between mean blood pressure in the carotid (▲) and in the femoral (△) arteries and juxtaglomerular rating

dures The haemodynamic conditions to which these kidneys have been exposed are illustrated in Fig 8 from which moreover the relation between the mean pressure in the carotid and femoral arteries and the juxtaglomerular rating may be read The drawing at the top of each column—a b c and d—illustrates the experimental situation concerned i e the position of the studied kidney in relation to the coarctation if any The mean blood pressure in the carotid and femoral arteries is given in per cent of the preoperative normal mean blood pressure The values for juxtaglomerular rating were calculated on the basis of PAS as well as Bowie stained preparation Corresponding values of mean blood pressure and juxtaglomerular rating are given in the same vertical column in the figure and the dogs number at the bottom In column a—representing a normal group as all the studied kidneys had been removed prior to the induction of coarctation—the juxtaglomerular rating ranges from 1 2 to 1 7 All the mean blood pressures are 100 In column b the juxtaglomerular ratings range from 1 1 to 3 7 With the exception of one dog (T 25) all the values are definitely elevated (2 3–3 7) compared with the normal values in column a Corresponding to these elevated juxtaglomerular ratings the mean blood

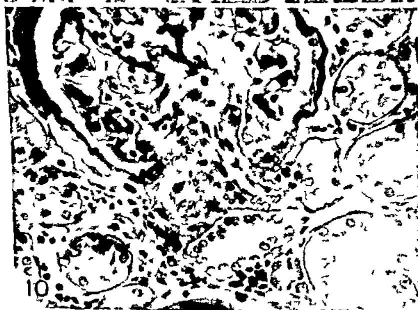
pressures are raised Dog T 25 with a juxtaglomerular rating of 1 2 and 1 1 had—at the last measurement—a moderately elevated mean blood pressure in the carotid artery and a lowered mean blood pressure in the femoral artery Its kidney was removed for investigation 11 days after the induction of coarctation when it died of haemorrhage from the operative site on the aorta Correspondingly the results of repeated blood pressure measurements revealed that in dogs with experimental coarctation and with a kidney peripheral to the coarctation the increase in the mean blood pressure usually appears a few weeks after the coarctation has been induced In the remaining dogs of column b all of which had high juxtaglomerular ratings and elevated mean blood pressures the kidneys had been examined 5–10 months after the induction of coarctation Column c gives the juxtaglomerular ratings in cervical kidneys from 2 dogs with coarctation in which the other kidney had been left in the abdominal site The values are normal i e 1 3–1 5 One of these dogs had a considerably elevated mean blood pressure in the carotid and femoral arteries The other one (T 25—see above) had moderately increased mean blood pressure in the carotid artery but a lowered mean blood pressure in the femoral artery Column d gives the juxtaglomerular ratings (1 5–2 2) in the cervical kidneys of dogs with coarctation whose abdominal kidney had been removed None of the mean blood pressures was elevated

Histological examination of the kidneys showed incidentally in 6 out of 14 abdominal kidneys small interstitial lymphocytic and plasma cell infiltrations quite considerable in one (T 30) In 3 (T 19 24 and 28) out of 12 cervical kidneys there was severe acute interstitial inflammation with abscess formation (pyelonephritis) All the remaining 9 cervical kidneys exhibited focal parenchymal atrophy and interstitial fibrosis with mild chronic inflammatory reaction One of these 9 kidneys (T 27) showed severe hydronephrosis three (T 16 25 and 26) mild arteriosclerotic changes with medial hyperplasia in the interlobular arteries and arterioles The remaining 5 cervical kidneys (T 20 21 22 29 and 30) showed no abnormalities apart from the focal parenchymal atrophy interstitial fibrosis and inflammatory reaction Fig 9)

DISCUSSION AND CONCLUSION

In 1939 *Goldblatt* and his co workers constricted the thoracic aorta of dogs by a modified renal arterial clamp The experiments failed as the dogs died of haemorrhage but it has been demonstrated by subsequent authors that when the thoracic aorta of dogs is sufficiently constricted the mean blood pressure central to the constriction will increase to hypertensive values in a few weeks Peripheral to the coarctation the mean blood pressure—low immediately after the induction of the coarctation—will increase in the course of the same period to normal or slightly elevated levels Direct measurement of flow and renal





Figs 9–10

Fig 9 Area f om the cervical kidney of dog T 22 h w ng f al atrophy of the parenchyma fibrosis and interstitial inflammator rea tion PAS × 48

Fig 10 Juxtaglomerular apparatus with hyperplasti rt l without granulation in the cervi al kidney of a dog (T 30) with a no mal mean blood pressure PAS × 400

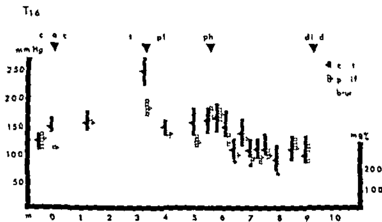


Fig. 2

Sequence of the experimental procedures, the time intervals and the results of determination of blood urea and blood pressure in 5 dogs (See also Figs. 3-6)

ated gross atherosclerotic changes. Table 1 lists the thickness of the aortic wall and the histological findings. In 4 of the operated dogs there was moderate proliferative intimal thickening central to the coarctation, never peripheral to it (Fig. 7). One control dog had mild intimal proliferation in the ascending as well as the descending aorta. Lipid deposits *in the vessel wall* were not found in any dog. *Fairly mild medial fibrosis* was demonstrated central to the coarctation in 4 of the operated dogs, peripheral to it in one. One control dog had slight medial fibrosis only in the ascending aorta. The configuration of the elastic lamellae was normal in all the dogs. The total number of elastic lamellae varied somewhat from dog to dog, presumably because they were mongrels, but in all the dogs except in one control it was greater in the ascending

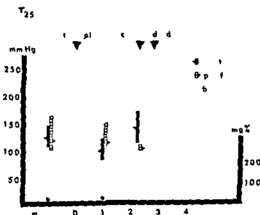


Fig. 3

(For text see Fig. 2)

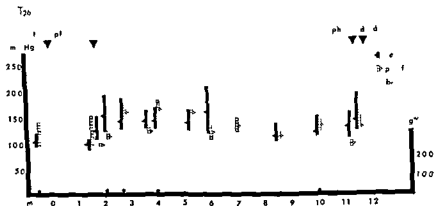


Fig 4
(For text see Fig 2)

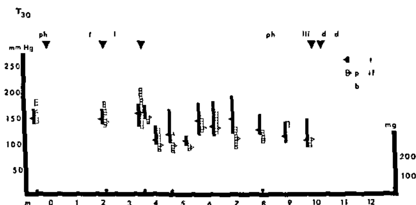


Fig 5
(For text see Fig 2)



Fig 6
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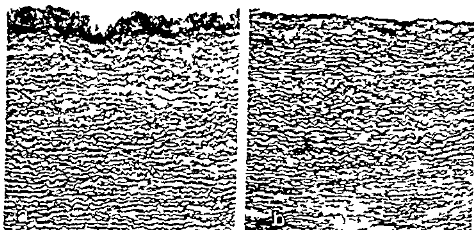


Fig 7

Intimal hyperplasia in the aorta central (a) but not peripheral (b) to the coarctation Normal configuration of elastic lamellae T 26 orcein × 48

TABLE 1

Mural Thickness and Histology of the Ascending and Descending Aorta (Centrally and Peripherally) in 5 Dogs with Coarctation and in 5 Control Dogs

	No	Thickness of aorta (mm) asc	Thickness of aorta (mm) desc	Intimal proliferation (0—++) asc	Intimal proliferation (0—++) desc	Medial fibrosis (0—++) asc	Medial fibrosis (0—++) desc	Number of elastic lam asc	Number of elastic lam desc	Number of elastic lam per field asc	Number of elastic lam per field desc
coarctation	T 16	3 0	2 0	0	0	+	+	148	128	17	21
	T 25	3 0	1 3	+	0	0	0	137	100	17	22
	T 26	3 5	1 5	++	0	+	0	159	117	18	22
	T 29	2 7	1 5	++	0	++	0	150	118	16	24
	T 30	2 0	1 5	+	0	++	0	103	90	21	23
control	A	2 5	2 0	0	0	0	0	152	120	20	18
	B	2 0	1 7	0	0	+	0	72	65	18	18
	C	2 0	1 5	+	+	0	0	74	68	21	19
	D	2 0	1 7	0	0	0	0	77	70	20	19
	F	2 0	1 7	0	0	0	0	92	9	18	21

than in the descending aorta (A direct comparison of the total number of elastic lamellae in the aortae of dogs with coarctation and in controls was not possible because of differences in the size of the dogs) On the other hand the relative number of elastic lamellae (mean number per field with a dry lens objective 45 1 subintimally centrally and sub adventitially in the media) was greater in the aortic wall peripheral to the coarctation in all 5 operated dogs (Fig 8) but in only one of the controls Correspondingly there was a more ample quantity of acid mucopolysaccharides (metachromasia staining with Astra blau) in the

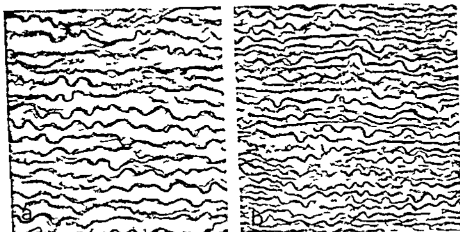


Fig 8

Density of elastic lamellae in the aorta central (a) and peripheral (b) to the coarctation T 29 orcein × 190

aortic wall central than peripheral to the coarctation (Fig 9) Since each aorta was fixed *in toto* this difference must be real—not caused by differences in fixative or fixation period The number and size of smooth muscle cells in the aorta were estimated to be the same central and peripheral to the coarctation and there was no difference in the amount of PAS positive substance No calcium deposits were found in the aortic wall

In other words there was an increased difference in the mural thickness of the ascending and descending aorta in the dogs with experi



Fig 9

Metachromatic ground substance in the aorta ce t l an l peripheral (b) to the coarctation T 29 toluidin l l 190

mental coarctation This increase was due predominantly to an accumulation of acid mucopolysaccharides in the media of the aortic wall central to the coarctation In addition intimal proliferation and medial fibrosis were more common in the aorta central to the coarctation than in the ascending aorta of control dogs selected at random The relation between these changes of the aortic wall and the duration of the experimental coarctation cannot be deduced from the present investigation However even the dog T 25 that died 2 weeks after the induction of the coarctation showed an accumulation of acid mucopolysaccharides in the media central to the coarctation but no fibrosis

DISCUSSION

The aortic changes found in the experimental coarctation in dogs corresponds approximately to the changes of human aortae exhibiting congenital coarctation of the adult type (*Heath & Edwards* 1959) The mural thickness is usually at a maximum above the coarctation and the configuration of the elastic lamellae is normal In several cases of adult coarctation however there are aneurysms above as well as below the constriction In *Abbott*'s opinion (1928) these cases as well as cases showing rupture without aneurysm are due to congenital weakness of the aortic wall

Dunnill's (1959) results however are to some extent at variance with the present ones In his 9 cases the aortic wall was usually thicker distal to the coarctation whereas the number of elastic lamellae in the media was greater proximally in 8 out of the 9 cases an increased quantity of acid mucopolysaccharides was found in the thickened media However it can hardly be expected that experimental coarctation is fully comparable with the spontaneous human condition in which congenital abnormalities of the aortic wall on either side of the stenotic areas may be operative

Studying the aortic changes in rats with experimental hypertension (unilateral renal ischaemia) *Margarey* (1957) found pronounced medial changes with cartilaginous metaplasia in 14 5 per cent of the rats whereas *Crane* (1962) in rats rendered hypertensive by nephrectomy 11 desoxycorticosterone + NaCl was unable to demonstrate an increased uptake of labelled sulphate in the aortic wall and thus presumably also no formation of sulphated mucopolysaccharides From arteriosclerosis research however it is known that sulphated mucopolysaccharides may accumulate in the aortae of experimental animals given adrenaline (*Berthelsen* 1961 *Lorenzen* 1963) and that with advancing age the composition of mucopolysaccharides in the media of the human aorta changes (*Dyrbye* 1959 *Clausen* 1964) These medial changes are an important link in the pathogenesis of aortic arteriosclerosis and are assumed by many students of arteriosclerosis to precede the intimal changes (*Thoma* 1923 *Berthelsen* 1964 *Lopes de Faria* 1965)

That our dogs did not exhibit intimal lipidosis and thus not full blown atherosclerosis must be viewed on the background of the fact that in rabbits *Bronte Stewart & Heptinstall* (1954) could not induce typical atheromatous changes unless the rabbits were simultaneously fed ample cholesterol Hypertension alone could not induce intimal changes in the rabbits

As emphasized by *May & Kaplan* (1961) coarctation of the aorta is a naturally occurring experiment illustrating the significance of hypertension to atherosclerosis In analogy the experimental coarctation described in the present paper seems to be a very well suited experimental model for studying the influence of haemodynamics upon the development of arteriosclerosis

SUMMARY

The histological changes in the aorta central and peripheral to an experimental coarctation were studied in 5 dogs All showed thickening of the aortic wall central to the coarctation mainly because of an accumulation of acid mucopolysaccharides in the media

These changes are in fair agreement with the findings in human coarctation of the adult type and are due to altered haemodynamic conditions The results might indicate that the elevated pulse pressure above the coarctation is a more important factor in this connection than the mean blood pressure

The significance of experimental coarctation in arteriosclerosis research is emphasized

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A QUANTITATIVE STUDY OF MAST CELLS IN APPENDICITIS IN MAN

By

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Received 23.xi.66

It was pointed out already by *Ehrlich* (1877) that mast cells were more numerous in the lower part of the gastrointestinal canal. *Samsonow* (1909) assumed that there was a special type of mast cell in the intestinal canal and that these cells were very numerous on the appendix wall. Mast cells of the normal type were encountered by *Weill* (1920) dispersed profusely throught the canal. *Michels* (1923), *Jordan* (1926) and *Bolton* (1933) reported a high mast cell count in the tunica mucosa and submucosa. A correlation was established by *Arvy & Quivy* (1955) between the mast cell count and the histamine content determined by *Feldberg & Harris* (1953) in the wall of the canine intestine. *Lindholm* (1959, 1960) performed quantitative determinations in the wall of the gastrointestinal canal of fetus, man, hamster and rat. A significant decrease was seen in the mast cell count in the lower part of the gastrointestinal canal of the human fetus. In man only the oesophagus and rectum had more profuse mast cells than the other parts of the gastrointestinal canal. The appendix contained 1 955 cells per cu. mm. In the hamster the highest mast cell count was in the appendix. A tendency towards a decrease in the mast cell count in the lower part of the gastrointestinal canal was established in both hamster and rat. A large number of mast cells were found by *Godlewski* (1960) in the ileocecal junction and appendix of man.

Ehrlich (1877) and *Westphal* (1880) noted that the mast cell count was lowered in acute and increased in chronic inflammations. It was reported by *Metchnikoff* (1892) that mast cells helped to remove toxins. *Maximow* (1904) confirmed that mast cells undergo regressive changes in inflammation. It was assumed by *Brack* (1925) that the increase in the mast cell count in chronic inflammatory conditions was indicative of increased connective tissue activity. *Nagayo* (1928) established that mast cells played no active role in acute inflammations but increased in number in chronic inflammations. On the other hand *Quensel* (1933) reported that there were no mast cells in the inflamed tissue itself but on the boundary between healthy and inflamed tissue. It was reported by *Maximow* (1904), *Ernst* (1926) and *Sanyal* (1959)

that mast cells react rapidly in inflammations degranulate and disintegrate *McGovern* (1957) established that mast cells may play a role in regulating inflammation and *Parish* (1964) was of the opinion that pharmocological substances released by mast cells are mediators of changes occurring in inflammation

MATERIAL AND METHOD

The material consisted of 49 appendices from human subjects 12 of them forming the control material[1] No *signs of acute appendicitis were seen in the control material* The specimens were taken from the midmost part of the appendix The material displayed clear signs of acute inflammation clinically grossly and *microscopically In the cases in* which inflammation was spread throughout the preparation the specimens were taken from its midmost part When gangrene or perforation was present they were taken from adjoining tissue Both sexes were represented The preparations were fixed in 4 per cent aqueous solution of *basic lead acetate The* were prepared in the usual way and cut in 10 µ sections Staining was done in 1 per cent toluidine blue aqueous solution for 10 minutes The mast cells were counted per sq mm in the tunica submucosa The mean mast cell count was calculated from 5 counted sq mm A Leitz binocular microscope with a field of vision of *0 066 sq* mm was employed The cells were counted in every other section and the number per cu mm was determined from *Floderus*'s (1944) formula The final mast cell values per cu mm were scaled *down by 44 7 per cent to eliminate the source* of error originating in the fixation of the preparations (*Lindholm* 1959)

RESULTS

Table 1 shows the results for the control material The minimum per sq mm was 23 the maximum 112 and the mean 55 2 cells per sq mm or 2184 per cu mm after scaling down

TABLE 1

The Mast Cell Count in the Tunica Submucosa of Human Appendix

Case No	Age years	Number of cells in 5×1 sq mm					Average per sq mm
1	4	56	65	47	66	69	60 6
2	10	45	61	58	56	61	56
3	19	94	71	81	48	56	70 0
4	39	56	57	45	50	49	51 4
5	41	28	23	48	36	37	34 4
6	44	38	32	32	49	[illegible]	41 0
7	48	39	46	33	32	38	37 6
8	49	40	60	67	59	41	59 4
9	49	109	89	112	79	72	92 0
10	51	64	39	33	43	45	44 8
11	53	90	94	76	72	75	81 4
12	61	54	46	42	37	24	40 6
						Mean	55
						Average per cu mm	3947
						Average per cu mm reduced by 44 7%	2184

[1] The control preparations came from the institute of Forensic Medicine University of Helsinki and the appendicitis preparations from the Department of Surgery Maria Hospital Helsinki

TABLE 2

The Mast Cell Count in the Tunica Submucosa of the Inflamed Human Appendix

Case No	Age years	Number of cells in 5×1 sq mm					Average per sq mm
13	7	112	112	140	138	125	125.5
14	7	19	19	21	21	18	19.6
15	10	55	84	70	52	60	64.2
16	11	45	63	56	44	40	49.6
17	11	69	67	52	69	62	63.8
18	11	39	41	47	35	37	39.8
19	12	37	33	24	30	25	29.8
20	13	78	59	88	64	75	72.8
21	16	36	41	50	46	38	42.2
22	17	25	27	22	16	27	23.4
23	19	67	36	47	40	26	43.2
24	24	27	20	18	21	34	24.0
25	25	13	8	10	11	7	9.8
26	27	28	25	45	32	33	32.6
27	27	51	56	57	59	54	55.4
28	28	68	70	62	67	73	68.0
29	34	43	28	25	39	28	32.6
30	35	23	18	17	21	14	18.6
31	36	33	31	31	36	41	34.4
32	6	79	107	114	75	69	88.8
33	16	43	47	39	45	50	44.8
34	25	30	31	19	25	27	26.4
35	26	50	57	60	60	41	53.6
36	26	37	41	34	28	35	35.0
37	29	24	32	21	39	33	29.8
38	31	38	28	33	36	30	33.0
39	36	47	76	73	60	58	62.8
40	48	83	110	113	64	81	90.2
41	56	18	40	46	32	48	36.8
42	10	80	61	59	78	78	71.2
43	21	77	46	56	54	64	59.4
44	25	39	54	36	60	46	47.0
45	26	32	36	46	35	38	37.4
46	39	41	66	66	60	56	57.8
47	65	59	68	56	52	74	61.8
48	66	66	78	50	74	59	65.4
49	67	49	54	43	41	44	46.2
						Mean	48.6
						Average per cu mm	3471
						Average per cu mm reduced by 44.7%	19 0

Table 2 gives the mast cell counts in the inflamed appendix The results can be divided into three groups Cases 13–31 are an example of moderate appendicitis with injection oedema and incipient gangrene The minimum per sq mm here was 7 and the maximum 140 cells mean 44.7 cells per sq mm Cases 32–41 show signs of appendicitis with actual gangrene and perforation The minimum cell count per sq mm was 18 and maximum 114 cells mean 50.1 Cases 42–49 display signs of moderate appendicitis with earlier acute attacks or periappendicular abscesses in the history The minimum mast cell count per sq mm

was 32, the maximum 80 and the mean 55.8. The mean for all the cases of appendicitis was 48.6 mast cells per sq.mm. or reduced 1920 per cu.mm. The mean age of the control material was 39.8 years and of the investigation material 26.7 years.

DISCUSSION

All appendicectomised patients displayed clinically typical signs of acute appendicitis. The preparations showed signs of inflammation both macroscopically and microscopically. In an acute inflammation tissue oedema with a subsequent increase in tissue volume is seen. This volume growth was not taken into consideration in the present work but tissue shrinkage in connection with the histologic preparation of the specimen was noted and was assumed to be the same for both normal and inflamed preparations. According to the study the cell count per sq.mm. for a normal appendix was 55.2 and for an inflamed appendix 48.6. Thus the decrease in appendicitis was insignificant.

Earlier workers such as *Maximow*, *Nagayo*, *Quensel*, *Ernst*, *McGovern* and *Sanyal* established a decrease in the number of mast cells in acute inflammation.

Taking the three appendicitis groups separately, it appears that the mast cell count was lowest, 44.7, in the most acute form of the disease; it was 50.1 in gangrenous and perforated appendicitis when a somewhat longer history can generally be assumed; the mast cell count was highest, *i.e.* 55.8, at the same level as in the control material, in the third group which can be regarded as a chronic phase about to become acute. This would seem to indicate that a decrease in the cell count occurs at the very beginning of an inflammation, *i.e.* degranulation. The tissue oedema which develops is probably due to the histamine released by the mast cells. Statistical analysis of the material showed that there was a significant ($P < 0.001$) decrease in the mast cell count in cases 13–31 compared with the control material. It was not possible to show this in the other groups. A counter reaction with possible new formation of mast cells and accompanying increased cell density may be assumed to take place as the inflammation continues. Or is the recovery of the mast cells to be attributed to possible increasing shrinkage of the oedematous tissue? Morphologically, no particular difference was seen between the mast cells counted in the control material and those in the investigation material. However, it is probable that mast cells play an important role in the regulation of an inflammation by yielding their products to the tissue (*McGovern*, *Parish*).

A way of establishing the cell density more exactly would be to take tissue preparations of equal size from the selected area and then count all the mast cells in the preparation as a whole.

SUMMARY

Mast cells in normal and inflamed human appendix were counted The preparations were fixed in basic lead acetate cut in 10 μ sections and stained with toluidine blue aqueous solution Cells were counted per sq mm in the submucosa and the cell count per cu mm was calculated from Floderus s formula

The control material displayed 2184 mast cells per cu mm the investigation material 1920 A significant decrease in the cell count was established right at the beginning of an acute inflammation Increasing mast cell density was seen in the course of the inflammation The situation is discussed in detail

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DIURNAL VARIATIONS IN SPONTANEOUS BODILY ACTIVITY IN HAIRLESS MICE MEASURED WITH ACTIVOGRAPH

By

OLAV HILMAR IVERSEN ELLEN FAGERENG ROLF BJERKNES
and GEORGY VERLY

Received 16.1.67

It is well known that the incidence of mitoses in mouse epidermis displays a circadian rhythm The diurnal variations in the epidermal mitotic count is possibly connected to the variation in spontaneous bodily activity It has been suggested that the blood concentration of adrenaline plays an important role (*Bullough* 1947–48) At our Institutes we are studying the kinetics of cellular proliferation in the epidermis of the hairless mouse in relation to carcinogenesis and we felt a need to know the rhythms of spontaneous bodily activity in our strain of hairless mice kept under the ordinary conditions in our laboratory

MATERIALS AND METHODS

Many methods of measuring the physical activity of small animals have been described previously (see for instance *Mann 1950* and *Wulff Rasmussen 1953*) However these methods have not provided measurements that are truly representative of the spontaneous activity They have not registered activity due to eating grooming or other such limited movements Some of the so called tambour mounted cages are very sensitive but the results are usually given in graphical recordings which are difficult to analyse quantitatively

We have built an activity recorder on the tambour mounted cage principle in which all activity is integrated over fixed periods Fig 1 shows the cage which rests upon four small balloons that are moderately inflated with air The apparatus was mounted in such a manner that it was in no way affected by vibrations in the building A rubber tube from each of the balloons is connected to a common membrane box and movements of this membrane are transformed into an electrical signal

The apparatus is shown schematically in Fig 2 The activity detector can be divided into a mechanical a pneumatical and an electrical section The bodily activity of the mice moves the cage on the air cushions The vertical components of these movements are proportionally transformed to a voltage by the detector Only signals within a certain frequency range are accounted for The upper limit of this frequency bands is determined primarily by the mass of the cage and the spring constant of the balloons

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This investigation has been aided by a grant from *The Jane Coffin Childs Memorial Fund for Medical Research* and *The Anna Fuller Fund*



Fig 1
The plastic cage with two hairless mice The cage is resting on 4 balloons moderately inflated with air In the background to the left is the membrane box

$$f_{upper} = \frac{1}{2\pi}\sqrt{\frac{k}{m}}$$

Where m = mass and k = spring constant

The lower limit is determined by the volume of the membrane box and the restriction of the derivating valve in the pneumatic section

$$f_{lower} = \frac{1}{2\pi} \cdot \frac{1}{RV}$$

Where V = volume of the membrane box and R = restriction of the derivating valve

By trial the system was so adjusted that neither the upper nor the lower limites were of practical importance fo the readings

In the RMS detector (RMS means Root Mean Square) the signal is ectified and an output proportional to the effective value of the input is generated This signal is thus also approximately proportional to the movements of the mice The *Integrator* is an operational amplifier coupled as an integrator The output is the time integral of the input after the last reset signal This output is the impuls (I) of the corresponding force (F) viz $I = \int_0^t F\,dt$

The *Encoder* transforms the data f om the analogue t d gi l f rm

At present we use a potentiometer recorder with a c l di c w th l na y contact pattern The *Time* consi ts of a synchrone motor w n n t fixed time intervals gives trigger signals to the tape punch and reset th ntegrat r The results are punched out on standard 8 hole paper tape On the n t pc dd tional nformation and comments can be *recorded* manually The pap ape can l ter be u ed directly as input to a d gital computer and many different l ulations can easily be perf rmed

The mice were kept in plastic cages and wer f i a l day between 1200 and 1400 hr (MET) The mice were given a complet t llet form and water ad libitum There was always some f od and wat c ble in the cage The animal

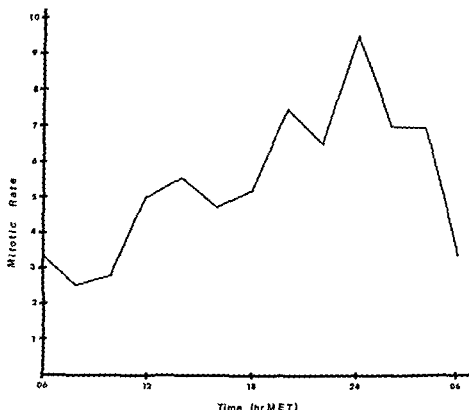


Fig 4

Circadian rhythm in average mitotic rate by the Colcemid method The rate is expressed as number of mitoses per 6 6 mm interfollicular back skin epidermis per hour

SUMMARY

The paper describes a device for measuring the physical activity of small animals kept under ordinary laboratory conditions

With this apparatus the diurnal variation in spontaneous bodily activity in hairless mice is registered

The technical advantages of the system is discussed The results are discussed with special emphasis on a possible correlation between physical activity and mitotic rate in the epidermis

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HISTOCHEMICAL STUDIES ON BRAIN PHOSPHATASES IN EXPERIMENTAL LEAD POISONING

By

ARNE BRUN and ULF BRUNK

Received 7 xii 65

Lead poisoning gives rise to dramatic neurological symptoms and yet in routine microscopy these cases usually show only minimal pathological changes in the brain such as patchy nerve cell degeneration and slight vascular endothelial reaction (*Pentschew* 1958) These were the alterations revealed in a 2 year old girl (O 39 66 Institute of Pathology Lund) dying from acute lead intoxication owing to a lead button arrested in her stomach

The effect of lead obviously has to be demonstrated by other means Since lead belongs to the heavy metals which are known to affect the enzymatic activity and since neurons and vessels showed changes it might be of interest to study some enzymes in these structures

As no useful material was left from the case mentioned above the problem was studied experimentally Unspecific alkaline and acid phosphatases in the vessels and neurons respectively were investigated in white rats poisoned with lead

MATERIAL AND METHODS

Twenty nine white male and female rats (Sprague Dawley strain) 6 weeks old and weighing about 190 g at the beginning of the experiment were taken from six litters They were fed on a standard diet consisting of commercial pellets and water ad libitum Eighteen animals received on each injection 10 or 20 mg water free $Pb(NO_3)_2$ per 100 g body weight solved in aqua redest in an amount to adjust the administered volume to 1 ml In all 100 to 200 mg $Pb(NO_3)_2$ per 100 g body weight was given The animals were injected subcutaneously on consecutive days except for Sundays a new site of injection being chosen every day to avoid necroses and scarring possibly interfering with resorption of the solution The controls the remaining eleven rats were given 1 ml saline subcutaneously

The animals which received lead had a greater weight increase than the control animals in spite of loss of subcutaneous fat. This may be due to the larger amount of faeces found in the bowel

The animals were decapitated the day after the last injection except for two killed after 9 and one after 15 days The brain was removed immediately and two slices [illegible] mms wide were taken one through the basal ganglia—hippocampus and one through the central portion of cerebellum—pontine nuclei from each animal at identical sites The slices were immediately frozen in solid carbon dioxide or a mix

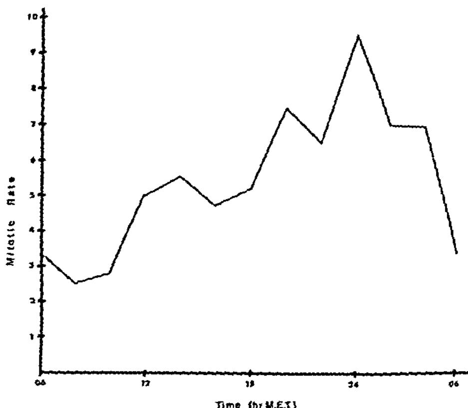


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By

Arne Brun and Ulf Brunk

Received 7 xii 66

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As no useful material was left from the case mentioned above the problem was studied experimentally Unspecific alkaline and acid phosphatases in the vessels and neurons respectively were investigated in white rats poisoned with lead

MATERIAL AND METHODS

Twenty nine white male and female rats (Sprague Dawle [illegible] t [illegible] ecl s old and weighing about 170 g at the beginning of the experiment [illegible] The were fed on a standard diet consisting of com n [illegible] ad libitum Eighteen animals received on each injection 10 [illegible] 20 m [illegible] (NO$_3$) per 100 g bodyweight solved in aqua redest [illegible] the administered volume to 1 ml In all 100 to 200 mg Pb(NO [illegible] ght was given The animals were injected subcutaneou [illegible] except for sundays a new site of injection being chosen e [illegible] and scarring possibly interfering with resorption of the [illegible] remaining eleven rats were given 1 ml saline subcutaneous !

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ture of propane propylene chilled with liquid nitrogen and cut at —20 C in a cryostat (Dittes Duspiva) in 10 μ thick sections transferred to cover glasses

Sections to be stained for enzymes were fixed for 30 min in acetone at —20 C in the cryostat and thereafter for another 30 min in acetone at room temperature Sections to be used for routine staining were while still wet fixed at room temperature in a mixture containing ethyl alcohol acetic acid and formalin (85 5 10)

For the demonstration of acid and alkaline unspecific mono phosphatases a simultaneous coupling azo technique was employed using Naphtol AS TR phosphatase as a substrate (*Burstone* 1958)

Acid phosphatases were demonstrated at pH 5 0 with fresh diazotised pararosaniline (*Barka & Anderson* 1963) A 0 1 M tris maleate buffer was used The sections were incubated for one hour at room temperature and at 37 C

Alkaline phosphatases were demonstrated at pH 9 2 with Fast Violet LB (Sigma) as a diazosalt The buffer was a 0 1 M tris maleate The sections were incubated for 20 min at room temperature

After staining the sections were washed carefully and some counterstained for 45 sec in Mayer s Haematoxylin All sections were mounted in plasdone C (*Burstone* 1957)

Alternate sections were used for routine stainings such as Nissl Mahon for myeline and Haematoxylin and Eosin after ethyl alcohol acetic acid formol fixation The sections then were dehydrated and mounted

In 10 cases those parts of the brains not used for cryostat cutting were analysed for lead with a spectrophotometric dithizon method in use at the Clinic of Occupational Medicine University Hospital of Lund For valuable assistence with the analysis we owe thanks to Dr B Fristedt engineer Övrum and colleagues

Sections from experimental and control animals were incubated in the same dish Changes in enzyme activity of the experimental animals were evaluated in reference to the over all activity in the control animal In addition localization of the activity of acid phosphatases within the cell and in different cell groups was studied and compared with that of control animals

The alkaline phosphatase present mainly in capillaries and choroid plexus but also found in the central gray matter was evaluated according to the over all activity The acid phosphatase present mainly in nerve cells of the hippocampus basal ganglia pontine nuclei cerebral cortex and purkinje cells was judged in the same manner with special attention paid to the two last mentioned structures

RESULTS

Routine histology (Mahon Nissl Haematoxylin and Eosin) showed mild to insignificant changes of the type referred to in the introduction namely discrete focal loss of neurons in the cortex The surrounding microglia showed a moderate proliferation and the endothelial cells in capillaries and precapillary vessels were slightly swollen There were no convincing signs of brain oedema

Quantitative lead analysis of 10 animals revealed a significant increase in lead in the brain as well as in other organs (liver kidney blood) No lead was found in the controls

Animals killed the day after cessation of lead administration showed a slight decrease in alkaline phosphatase activity in the capillaries in all but one animal where the activity tended to be stronger Simultaneously the activity of the acid phosphatase in the neurons compared to that in controls appeared to be increased in all 15 animals killed the day after cessation of lead administration In rats killed 9 days or more after the last lead injection the enzymatic activity did not differ from that in controls Restoration to normal activity tended to be somewhat slower for acid phosphatases than for alkaline The impression of in

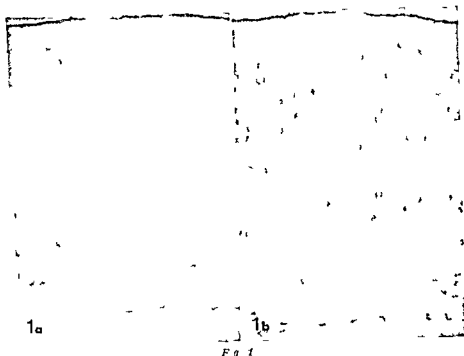


Fig 1

Parasaggittal neocortex × 63 acid phosphatase naphtol AS TR diazotated pararos aniline a control b lead poisoned rat Note in b increased neurophil staining esp in cortical layers II IV and increased cellular staining esp in layer III and V compared to a

creased activity of acid phosphatases *e g* in the parasaggittal neo cortex in lead poisoned animals on closer inspection was found to be due to a stronger staining of the neurophil especially in cortical layers II–III and of the neurons in layers III and IV (Fig 1 a + b) The staining of the neuron cytoplasm was granular in controls and in poisoned animals in addition more diffuse (Fig 2 a + b) Especially the nerve cells of layer V stood out as densely stained (Fig 2 a + b) In the neurons the stained granules tended to be coarser and clumped together in a narrow perinuclear zone of the cytoplasm (Fig 3 a + b) These changes enhanced the laminar distribution of cortical neurons (Fig 1 b compared to 1 a) In the purkinje cells these differences were much less marked

DISCUSSION

Located in the lysosomes the *acid phosphatase* is assumed to play a role in the production of energy and in the protein synthesis and intra cellular digestion (*Bejdl* 1955 and *De Duve* 1965) An increase of the histochemically detectible enzyme activity is not only in axonal reaction but also in electro shock liver coma (*Adams* 1958) and high age with senile degeneration (*Josephy* 1949)

In a study on autolysis in the brain a shift from bound to free activity was noted the total activity remaining constant (*Anderson* 1965) This shift may be due to rupture of lysosomes and release of enzyme into the cytoplasm Histologically this may produce a change from normal granular pattern to a more diffuse staining of the cyto plasm In autolysis Andersen also noted a perinuclear aggregation of granulae

The apparent increase of acid phosphatase activity and diffuse stain ing of the cytoplasm found in the present study may be due to an in crease of enzyme or a consequence of liberation of the enzyme into the cytoplasm from ruptured lysosomes more of the cytoplasm becoming stained than normally We also found the above mentioned perinuclear aggregation of stainable granules

There are thus similar changes in acid phosphatase distribution in autolysis and lead intoxication In the latter condition however the changes are reversible and may be interpreted as a sign of degeneration or an increased metabolic effort on the part of the nerve cell analogous to what is seen in the regenerating neuron in the axonal reaction In this situation however there may be no real difference between regenera tion and degeneration the latter being an introductory step and ne cessary prerequisite for ensuing regeneration The apparently increased activity may thus be compatible with a reversible degenerative process in the neuron vaguely suggested by routine histology

The *alkaline phosphatase* is assumed to partake in the glucose re sorption in the brain (*Stadtman* 1961) and also collagen formation in the body Its role in the central nervous system however is far from clear

The reduced activity found in the present study may indicate a toxic inhibition produced by the lead which is known to be toxic to vessels (*Pentschew* 1958) With regard to the dependence of the brain on glucose this should be important for the metabolism of the CNS This hypothetic block may be an important link in a chain of events leading to interference with nerve cell function in lead intoxication It may also be placed in conjunction with the known microscop c changes revealed by the vessels in lead intoxication *viz* endothelial hyperpla a and arteriosclerotic like changes

The normalization of the changes in enzyme act v ty r c ssation of lead administration found in the present stu indi s that enzy

---

Figs 2 3

Fig 2 Parasaggittal neocortex la r V × 100 a phos t c a Fig 1 a ontrol b lead poisoned rat Note increased sta ng pr t es f nerve cells in b compared to a

Fig 3 Basal pontine nucl ar nerve cells × 4 acid pl phatase as in Fig 1 a control b le l po soned rat Granules c nc ntr ted to p rinuclear zone of cytoplasm in b

matic damage caused by the lead was reversible This is in agreement with the restitution of many clinical symptoms

CONCLUSIONS AND SUMMARY

Routine light microscopy shows minimal pathological changes in acute experimental lead poisoning in rat in spite of accumulation of lead in the brain tissue

Histochemically apparently reversible changes appear consisting of slight reduction in the activity of alkaline phosphatase in the vessels but *apparent increase of the activity and changed distribution of acid phos*phatases in the neurons The effect of lead is more easily demonstrated by histochemical methods than by routine microscopy

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5-HYDROXYTRYPTAMINE IN NORMAL AND SECTIONED RAT SCIATIC NERVE

By

NILS ERIK ANDÉN and YNGVE OLSSON

Received 24 x 66

Mast cells are present both in the epineurial and perineurial coats and in the connective tissue of the endoneurium in peripheral nerves of the rat (*Enerback et al* 1965 *Olsson* 1965 1966) Rat mast cells have been found to contain several highly active biological substances The capacity of these cells obtained from other tissues to synthesize and store not only heparin and histamine but also 5-hydroxytryptamine (5-HT serotonin) has been demonstrated by several investigators (for ref see *Riley* 1959 *Smith* 1963 *Padawer* 1963 *Uvnäs* 1964 *Selye* 1965 *Bloom* 1965) There are strong reasons to believe that the same findings hold true for the mast cells in peripheral nerves of the rat (*Olsson* 1965)

Recently it has been found that sectioning of the rat sciatic nerve is followed by a very large numerical increase of endoneurial mast cells throughout the distal part of the operated nerves (*Enerback et al* 1964 1965) The aim of the present study was therefore to study the changes in 5-HT in the part of the sciatic nerve distal to a transection using biochemical methods

MATERIAL AND METHODS

Eleven groups each consisting of four male Sprague Dawley rats were used Each group comprised rats of the same age weighing between 200 and 475 grams at the end of the experiments All determinations of 5 HT were done on separately pooled material from the right and left sciatic nerves

Control rats Three groups of rats were used as controls The rats were sacrificed by decapitation without anaesthesia and the sciatic nerves from tuber ossis ischii to fossa poplitea were removed immediately

Experimental rats In eight groups the right sciatic nerve of the rats was sectioned immediately below tuber ossis ischii under anaesthesia After 2 4 6 and 12 weeks respectively the rats were sacrificed by decapitation The sectioned nerves from the scar to fossa poplitea and the corresponding part of the non operated left nerves were pooled separately and then analysed for their content of 5 HT Both in control and experimental rats the nerves were dissected together with their epineurium and perineurium

Determination of 5 HT The 5-HT of the sciatic nerves was determined spectrofluorimetrically in 3 N HCl after cation exchange chromatography (*Bertler* 1961 *Andén & Magnusson* 1967)

This work has been supported by the Swedish National Association against Heart and Chest Diseases For technical assistance we thank Miss *Birgitta Nilsdotter Högberg*

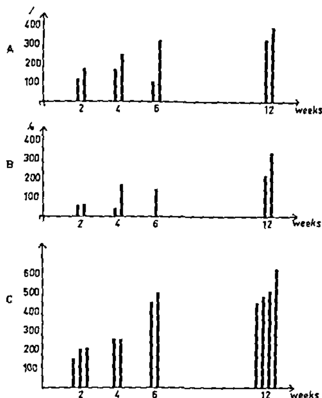


Fig 1

The content of 5 hydroxytryptamine (a) the concentration of 5 hydroxytryptamine (b) and the number of endoneurial mast cells (c) in the distal portion of the right sciatic rat nerve in per cent of that of the same portion of the left nerve The right nerve was sectioned at the time 0 Each column represents a single determination The columns at point 0 in A and B represent control determinations in two not operated rats × determination not performed

RESULTS

The distal portion of the left unoperated sciatic nerves contained 0 078 μg 5-HT per 4 nerves (s e m = 0 0145 n = 11) or 0 53 μg/g wet tissue (s e m = 0 045 n = 11) Any differences in 5-HT content or in weight between the left sciatic nerves taken from unoperated and operated rats were not observed

The distal portion of the right sciatic nerves in the unoperated rats contained about the same amount and concentration of 5-HT as that of the left nerve (Fig 1) After sectioning the 5-HT in the distal segment of the cut nerves displayed a marked and successive increase during the twelve weeks of investigation (Fig 1) Since the weight of the nerve distal to the lesion also showed an increase the concentration expressed in per cent did not rise to the same degree as the total content

The increase in number of endoneurial mast cells in the sciatic nerve distal to a lesion is shown in Fig 1 c (values from Enerback et al 1965)

The time course of the increases of endoneurial mast cells and of 5–HT is similar.

DISCUSSION

The present study revealed that normal rat sciatic nerves contain 5–HT. The cellular localization of 5–HT in peripheral nerve trunks has previously been studied with the fluorescence microscopic technique of Hillarp & Falck for monoamines (*Olsson* 1965). In the sciatic nerves of normal rats yellow fluorescence characteristic of 5–HT was observed in the mast cells found in the endoneurium and the surrounding sheaths. Thus it seems most likely that the major part of the 5–HT content in the nerves is derived from mast cells in the endoneurial, the perineurial and epineurial sheaths.

This study also demonstrated that nerve section is followed by a significant increase in the 5–HT content in the distal part 2 to 12 weeks after operation. A large increase in the number of endoneurial mast cells in sectioned nerves during the same period has been observed previously (*Enerbäck et al* 1964, 1965). These newly formed mast cells also give histochemical reactions typical for 5–HT (*Olsson* 1965). These observations strongly suggest that the increased level of 5–HT found in the distal part of sectioned nerves occurs in the numerous endoneurial mast cells present in the degenerating nerves. The finding of a good agreement between the time course of the increases in the 5–HT and the endoneurial mast cells also favours this assumption.

The increase of the 5–HT concentration was, however, smaller than the increase in the number of endoneurial mast cells. This difference may be due to the fact that the newly formed mast cells are smaller than the normal ones and therefore in all probability contain a smaller amount of 5–HT (*Enerbäck et al* 1964, 1965, *Olsson* 1965). The possibility also exists that the difference is due to different methods of calculation. The biochemical experiments were performed on the whole nerve while the number of mast cells were counted only in the endoneurium. Since the epineurium and perineurium normally contain at least as many mast cells as the endoneurium and as the increase in the number of mast cells after section appears to be restricted to the endoneurium, the increase should be smaller in the biochemical experiments.

SUMMARY

The content of 5–hydroxytryptamine in the rat sciatic nerve distal to a transection increased by about 300 per cent during a 12 week period following the lesion. The time course of this increase coincides with that previously obtained for the increase in number of endoneurial mast cells.

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CONGENITAL PRIMARY ADRENAL HYPOPLASIA

By

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Adrenal hypoplasia in association with severe malformation of the brain was described already by *Morgagni* Such adrenal hypoplasia has also been reported in combination with maldevelopment of the pituitary (3 8 14) In this anencephalic form the adrenals are small and their cortex is of adult type Such hypoplasia has however also been seen in otherwise normal children of mothers with a serum agglutinin incompatible with the infant s red cells (Rh incompatibility) and in children of elderly primiparae or of multiparae above 40 years and of mothers who had toxicosis of pregnancy (6)

During the last two decades some 10 cases of another type of adrenal hypoplasia have been reported This type is characterised histologically by a foetal cortex of cytomegalic type and differs also clinically from the anencephalic type

This paper is concerned with a new case of the latter type together with a survey of the literature and certain features suggesting that the condition is hereditary

REPORT OF CASE

The mother aged ?3 (born 1943) had been admitted to hospital because of tuberculosis in 1951 and had afterwards felt well Rh negative No abortion In 1964 she gave birth to a healthy boy She was now pregnant again and parturition was expected at the end of January 1966 Routine examination by a private practitioner in December 1965 revealed nothing remarkable On January 20 1966 she was delivered while on her way to hospital in an ambulance According to the ambulance orderlies the child had shown no signs of life and on arrival at hospital 5 to 10 minutes later the child was dead Tests for toxoplasmosis in the mother (dye test and complement fixation) were negative

Necropsy (98/66 Malmo) Boy crown heel length 39 cm Weight 1[illegible]0 g Skin around nose and mouth cyanotic The main findings were widespread haemorrhages with bilateral subdural haematomas and purpura of the skin soft meninges serosal surfaces surface of thymus and mucosa of the pharynx and urinary bladder The lungs were not aerated The testes were of normal appearance but were situated in the abdomen The pituitary was of normal gross appearance

It was soon noticed that the adrenals seemed to be missing Despite careful removal and thorough examination of all the internal organs no adrenals could be found The entire material in the region of the kidneys was therefore set aside for examination in serial sections No other abnormalities were found The umbilical cord contained three normal vessels The placenta showed both large and small infarcts

All of the internal organs except the spleen which weighed 8 g compared with a normal weight of 4 9 g ([illegible]) were small for total body weight

TABLE

Author	Age at onset of symptoms	Symptoms	Clin exam	Steroid therapy	Age at death
Sikl 1948	21 days	No appetite Diarrhoea Weight loss Pigmentation Bradycardia	Died on the day of admission to hospital	—	33 days
Geppert et al 1950	Newborn	Vomiting as in pyloric stenosis Pigmentation	17—KS in 24 hr urine 0 12 mg	+	20 months
Deamer et al 1950	Newborn	Vomiting as in pyloric stenosis Weight loss	Hyponatremia Hyperkalemia 0 60 mg	+	10 months
Mac Mahon et al 1957	14 days	Vomiting	Hyponatremia Hyperkalemia 17—KS 0 60 mg	+	3 months
Harlem et al 1957	16 days	Pigmentation	ECG as in hyperkalemia	+	33 days
Mitchell et al 1959 I	21 days	Vomiting as in pyloric stenosis	—	—	7 weeks
Mitchell et al 1959 II	21 days	No appetite Vomiting	Hyponatremia Hyperkalemia 17—KS 0 24 mg	+	Did well o
Boyd et al 1960 I	11 days	No appetite Vomiting	—	+	28 days
Boyd et al 1960 II	9 days	Vomiting	Died on the day of admission to hospital	+	12 days
Stempfel et al 1960 II	Case not published primarily				18 hours
Stempfel et al 1960 II	12 days	No appetite Vomiting Weight loss	Hyponatremia Hyperkalemia	+	Did well on
Lindgren 1966	—	Died from aspiration during delivery			Newborn

owing to the considerable loss of weight of the adrenals in association with postnatal involution

Though complete absence of adrenals has also been reported especially in anencephalia careful examination of some large series of

1

Body weight (g)	Wt of adrenals (g)	Missingor barely any z glom	Irreg foetal z	Cyto megaly	Hypo physis	Other endocr organs	Other malformations
3 700	not weighed 10 × 8 mm 12 × 8 mm	+	+	+	normal	normal	—
15 800	? 5 (1 5 + 1)	+	—	—	normal	no in formation	—
7 900	0 50 + 0	+	+	+?	no in formation		—
no in forma tion	1	+	+	+	not examined	normal	Slight aortic coarctatic
3 700	0 17 + 0 082	+	+	—	normal	normal	macrogenitosom
3 100	0 7?	+	+	+	normal	normal	—
ubstitution therapy 19ɔ9 1 year of age							
? ?50	0 6?	+	—	+	normal	normal	—
3 200	0 9	+	—	+	normal	normal	—
no in forma tion	—				no in formation		
ubstitution therapy 1960 3½ years of age							
1 780	Only micr	+	+	+	Macr normal	See de script on	—

anencephalia has invariably revealed the presence of adrenals (6) Except in a few cases with extensive malformation of the brain or of the lower trunk it must be seriously questioned whether the adrenals ever are really absent

Hypoplasia involves both adrenals in their entirety but particularly one of the two cortical zones which can be distinguished in the foetus In anencephalia (so called secondary type) the inner or foetal zone is very hypoplastic In so called primary or idiopathic adrenal hypoplasia the zona glomerulosa is most hypoplastic or missing But the preserved foetal zone often shows enlarged cells This type of hypoplasia is therefore known as the cytomegalic type Our case appeared to be of this type

A perusal of the literature revealed 11 cases assignable to this category (Table 1) All of the cases had been seen in boys including 3 pairs of brothers (4 13 18) This occurrence in brothers suggests the possibility that the condition may be hereditary

In primary or cytomegalic hypoplasia the weight of the adrenals at autopsy was always small and in all cases except one they weighed less than 1 g In that case (16) which was seen in a 20 month old boy (case 9) the adrenals weighed 2 5 g (normal weight about 5 g) In one case reported by Stempfel no adrenals were found post mortem That case had not been examined personally by Stempfel and it is not known how thorough the search for the adrenals had been

In the literature cases the patients lived for days to years Ours was the only case in which the patient died at birth Death was probably due to aspiration but the functional disorders due to adrenal hypoplasia might have been a contributory cause

In the other cases the manifestations of the disease appeared within the first 3 weeks of life in the form of loss of appetite vomiting loss of bodyweight and fairly rapid dehydration Some cases were clinically misinterpreted as pyloric stenosis and in 2 cases (7 and 13 case 1) explorative surgery was done because of such a provisional diagnosis In most cases the clinical picture resembled that of Addison s disease with hyponatraemia and hyperkaliaemia low concentration of 17 keto steroids in the urine and sometimes pigmentation of the skin Treatment with NaCl and steroids often produced improvement and control of the symptoms At 1 to 20 months however some of the patients suddenly went into shock and died At the time when the case reports appeared in the literature two patients aged 1 and 3½ years were still alive and doing well on substitution therapy

Of the 11 literature cases the reports included histological descriptions of the adrenals in 8 In most of these there was a broad band of cells resembling those in the inner zone in the normal foetus In half of the cases the zona glomerulosa was completely missing The others had only a very narrow outer zone The inner zone was built up of large cells not arranged in the normal trabecular fashion The cells were polygonal with abundant eosinophilic cytoplasm and round nuclei In most cases the adrenals contained some large giant cells with eosinophilic cytoplasm The diameter of the cells was twice that of normal cells and the nuclei were situated eccentrically Some cells were very

cular with prominent nucleoles while others were dense and hyper chromatic

The giant cells occurring in 6 of the 9 cases were in good agreement with those described by *Kampmeier* (11) He found single giant cells in the cortex of all 2 3 month old foetuses The older the foetus the fewer the giant cells Cytomegaly has been found in 6 5 per cent of new borns (5) and in 1 4 per cent of infants up to 4 months of age (1) In the latter series 6 out of 16 had various malformations Further *Beatty & Hawes* (2) found malformations in 9 out of 11 cases of cytomegaly and *Dhom* (6) in 2 out of 4 Cytomegaly in primary adrenal hypoplasia should probably be regarded as part of the maldevelopment In the above cases of cytomegaly the adrenals were of normal size in all except 1 Though this child was anencephalic we considered it justified to classify the disease as primary *cytomegalic* adrenal hypoplasia Cytomegalia which should not be confused with cytomegalic inclusion disease may be a manifestation of incomplete maturity or a return to embryonal cell formation

This together with the above mentioned occurrence of the condition in three pairs of brothers argues in favour of the theory that the mal development is of hereditary value If a woman has given birth to a child with adrenal hypoplasia she should be carefully watched during later pregnancies so that hypofunction if any of the adrenal cortex may be discovered and treated in time

SUMMARY

A baby boy born at term died from aspiration during delivery Gross examination post mortem disclosed no adrenals but histological examination of serial sections of retroperitoneal tissue revealed 2 very small adrenals The permanent cortex was very thin on the left side and practically absent on the right The foetal cortex was predominant and was built up of irregularly arranged eosinophilic cells and numerous giant cells The case was diagnosed as congenital primary hypoplasia (cytomegalic type) which should be distinguished from secondary hypoplasia with anencephalia Eleven cases were traced in the literature among these three pairs of brothers The condition therefore appears to be a hereditary anomaly which soon produces symptoms of cortical insufficiency Two of the above 11 patients aged 1 years and 3½ years and receiving substitution therapy are still alive

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INFLUENCE OF DIFFERENT ANTIGENS ON CIRCULATING LYMPHOCYTE POPULATIONS

By

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Received 13 iii 67

In a previous study a cyclical variation in the composition of the circulating lymphocyte populations in blood and lymph was found after a single subcutaneous injection of pertussis vaccine (*Ernstrom & Larsson* 1967a) A similar fluctuation in the number of immunocytes in the spleen has been reported after immunization with certain lipo polysaccharides (*Britton & Moller* 1966) In the present investigation the influence on the lymphocyte populations of two bacterial lipopoly saccharide antigens known to induce production of 19S antibodies was compared with that of two antigens known to initiate a rapid but short lasting production of 19S antibodies subsequently replaced by a synthesis of 7S antibodies (*Benedict et al* 1962 *Bauer et al* 1963) Furthermore the thymic export and import of blood lymphocytes during the primary response to the antigens was investigated by a comparison between lymphocyte content in afferent and efferent thymic blood

MATERIAL AND METHODS

Totally 3 5 male guinea pigs were used 14 of which were untreated normal ani mals The rest were divided into 4 main groups and were given a single intra peritoneal injection of one of the following antigens (number of animals and initial body weight in brackets mean ± S E)

Pertussis vaccine (commercial preparation of the State Bacteriological Laboratory Stockholm Sweden) in a dose of 2 5 ml per animal corresponding to 50×10^9 bacteria (67 245 ± 6 g)

Lipopolysaccharide extract from *E coli* (Lipopolysaccharide W *E coli* 055 B5 extracted by Difco Laboratories Detroit U S A) in a dose of 1 mg per animal (78 249 ± 4 g)

Sheep erythrocytes (fresh erythrocytes washed four times with saline and diluted to a 10 per cent suspension in 0 9 per cent saline) in a dose of 2 5 ml per animal (100 243 ± 2 g)

Salmonella typhi H (suspension of formalin killed *S typhi* of the strain I S 57 rich in the *H* antigen formalin 0 4 per cent) in a dose of 1 ml per animal corresponding to 14×10^9 bacteria (66 246 ± 4 g)

In each group 5 to 11 animals were investigated at different intervals after antigen administration The investigations were performed between 9 a m and 4 p m without any selection of the different group times All the animals used were kept under identical care during the experimental period and fed on cabbage turnips carrots and vitamin pellets Injection of the lipopolysaccharide from *E coli* resulted in death of a few animals 1 day afterwards The other antigens caused no mortality

The guinea pigs were anaesthetized with sodium Nembutal (2.5–5.0 mg in a 2.5 per cent solution i.p. per 100 g b.w.). The thymus was exposed and a thymic vein severed. Blood was collected in heparinized pipettes (Heparin® Vitrum, Stockholm, Sweden) for counting of white cells in a Bürker chamber and for classification of the lymphocytes by their mitochondrial content in supravital preparations. For the latter purpose slides were prepared with a mixture of Janus green B and neutral red in alcoholic solution (see *Cutting* 1957). A small drop of blood was applied to the slide, a cover glass was put on and sealed with wax. The preparations were immediately *examined in a light microscope at 1000 X magnification*. The lymphocytes (100 cells/sample) were registered in six classes: cells with 0–5, *6–10, 11–15, 16–20, 21–30 and >30 mitochondria. The mitochondrial content (MC)* is correlated to the size of the lymphocytes—low MC corresponds to small lymphocytes, high MC to large lymphocytes (Wiseman 1931, *Fichtelius & Larsson 1961, Ernström & Larsson 1963).*

The right carotid artery was cut near the origin of the thymic arteries and blood samples were taken for the same analyses as mentioned above. The artery was then ligated.

Finally, the thoracic duct was dissected free at its confluence with the left subclavian and internal jugular veins, using the technique of *Reinhardt & Yoffey* (1957). The duct was incised and a sample of lymph was collected for classification of the lymphocytes by their mitochondrial content according to the technique used *for blood. Afterwards the thymus* was taken out and weighed and the animals were killed.

For the white cell count in the Bürker chamber 25 mm³ of blood was diluted with 475 mm³ of Toisson's solution containing methyl violet for staining of the white cells. Totally 288 squares were counted in each sample, each square corresponding to 0.00625 mm³. *Differentiation was made between mononuclear and polynuclear cells.*

A mitochondrial content of 0–10, 11–20 and >20 mitochondria per lymphocyte is denoted as low, medium and high MC respectively. The results were analyzed statistically by Student's *t* test. The comparison between the number of lymphocytes of different categories in thymic vein and carotid artery blood was performed by *statistical analysis of all the differences in the individual animals.* The p values <0.05, <0.01 and <0.001 are denoted as almost significant, significant and highly significant, respectively.

RESULTS

The composition of the lymphocyte populations in blood and lymph of the normal guinea pigs is shown in Table 1. The difference in number of small lymphocytes per mm³ of blood from the thymic vein and from the carotid artery in the *normal animals* was *highly significant* (statistical analysis of the differences from the individual animals).

TABLE 1

Composition of the Lymphocyte Populations in Thymic-Vein Blood, Carotid Artery Blood and Thoracic Duct Lymph of Normal Animals. Mean ± S.E.

	No. of animals	Lymphocytes: Cells with low MC		Cells with medium MC		Cells with high MC	
		%	No./mm³	%	No./mm³	%	No./mm³
Thymic vein blood	14	*64.8±0.6*	*781±62*	*32.4±0.4*	*393±33*	2.8±0.3	31±4
Carotid artery blood	14	55.8±0.4	641±59	39.9±0.3	458±41	4.3±0.2	48±4
Thoracic duct lymph	13	28.5±0.6		62.9±0.7		8.6±0.2	

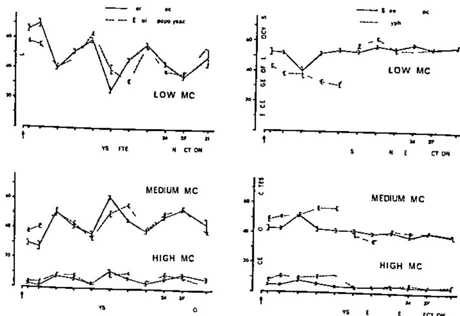


Fig 1

Percentage of lymphocytes with different mitochondrial content (MC) in blood from the carotid artery of guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi H* antigen The arrow indicates injection of antigen Mean ±S E

The antigen injections did not influence the normal gain in body weight (about 5 g per day) or the general condition of the experimental animals An exception was the *E coli* lipopolysaccharide which affected the condition of some animals on the first days after injection and also caused the death of a few animals

The absolute and relative thymic weights were not significantly changed after the injection of the different antigens

The granulocytes behaved very similarly after injection of the different antigens In all four experiments an increased number of granulocytes occurred one day after the injection After 3 days the number had fallen to about the normal level The granulocytes then varied in number with maxima at 9–12 and 21–24 days and minima between No significant difference was present between the number of granulocytes in the carotid artery and thymic vein blood

Pertussis Vaccine

The percentage and number of small blood lymphocytes (with low MC) increased and decreased cyclically as in a previous experiment when a subcutaneous injection of the vaccine in a lower dose was given (*Ernström & Larsson* 1967a) Maxima of percentages were noted 3 12 and 21 days after injection The percentage of medium sized and

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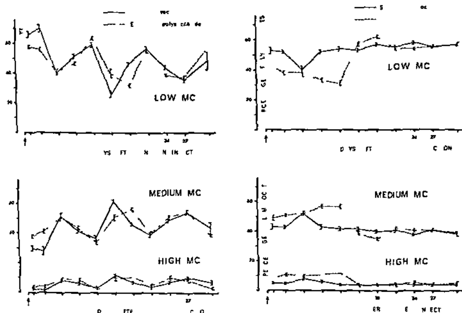


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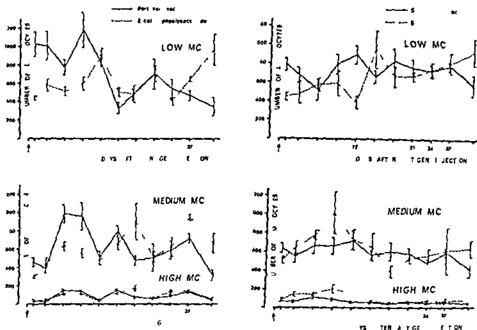


Fig 2

Number of lymphocytes per mm³ of blood from the carotid artery in guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi H* antigen The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) The arrow indicates injection of antigen Mean ±S E

large lymphocytes (with medium and high MC) also varied cyclically with minima when the number of small lymphocytes was maximal and *vice versa* (Fig 1) The number of lymphocytes per mm³ showed similar cyclical variations as the percentages (Fig 2) At 31 days after the *vaccine injection a decrease was recorded in all categories of lymphocytes*

The thymic veno arterial difference in number of small lymphocytes varied and 9 days after injection of the vaccine no difference was present An import of large lymphocytes into the thymus was demonstrated with significant values at 1 9 and 15 days after injection (Fig 3)

In the thoracic duct lymph as in the blood marked cyclical variations occurred in the relative frequency of the lymphocytes with low medium and high MC with the same periodicity as in the blood (Fig 7)

Lipopolysaccharide from E coli

A cyclical variation was recorded in the percentage of lymphocytes with low medium and high MC and in the number of these cells per mm³ of blood with maximal incidence and number of small cells at

the same time is minimal incidence and number of medium sized and large cells and *vice versa* (Figs 1 and 2) The cyclicity was similar to that seen after an injection of pertussis vaccine

The thymic veno arterial difference in number of small blood lymphocytes per mm³ of blood varied and 15 and 24 days after injection no mean difference was present (Fig 4) Concurrently with this interrupted thymic export of small lymphocytes a minimum number of such lymphocytes was noted in the blood (Fig 2) At 15 and 18 days a significant thymic export of medium sized lymphocytes occurred coinciding with the finding of a maximal number of such lymphocytes in the blood A significant import of medium sized and/or large lymphocytes took place 1 12 21 and 24 days after antigen injection (Fig 4)

In the thoracic duct lymph as in the blood a marked cyclical variation was found in the percentages of small medium sized and large lymphocytes The maximal and minimal incidence of the different categories of lymphocytes as a rule occurred at the same times as in the blood (Fig 7)

Sheep Erythrocytes

The percentage of small lymphocytes was decreased after the antigen injection with a minimal value at 6 days ($p < 0.001$) Correspondingly the percentage of medium sized and large lymphocytes was raised the increase also being maximal at 6 days ($p < 0.001$) These changes were found both in thymic vein and carotid artery blood From day 9 and onwards the percentage of all categories of blood lymphocytes remained on the level of that in the normal animals (Fig 1)

The number of small lymphocytes (with low MC) was not markedly changed after injection of the antigen The number of medium sized and large lymphocytes was slightly increased 6–12 days after injection (Fig 2)

As in normal guinea pigs a thymic veno arterial difference in the number of small lymphocytes was found in the immunized animals indicating an export of small lymphocytes from the thymus One day after injection of the antigen a large export was noted but after 3 6 15 and 18 days the export was almost significantly lower than after 1 day The medium sized and large lymphocytes were more frequent in the carotid artery blood than in the thymic vein blood indicating a thymic import of such cells during immunization (Fig 5)

In the thoracic duct lymph the percentage of small lymphocytes diminished after antigen injection to a minimum value 6 days later as in the blood The incidence of medium sized and large lymphocytes were correspondingly increased At 9 days and later the composition of the lymph was about normal as regards the lymphocyte population (Fig 7)

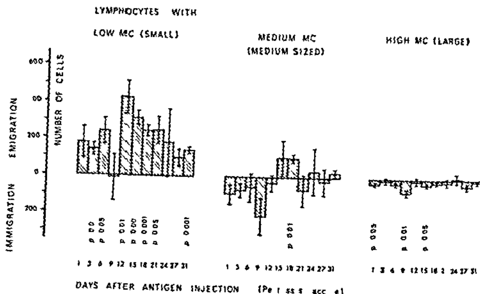


Fig 3

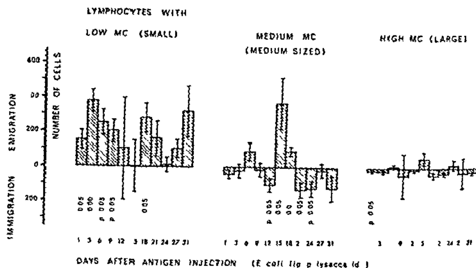


Fig 4

Figs 3–6

Thymic emigration and immigration of lymphocytes in guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi H* antigen The bars represent the difference between the number of lymphocytes per mm³ of blood from the thymic vein and from the carotid artery The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC) Mean ±S E

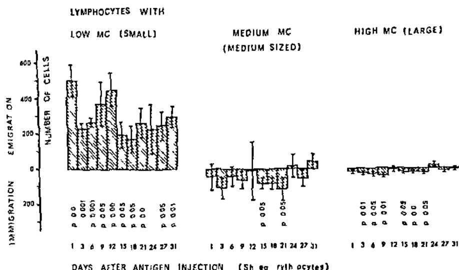


Fig 5

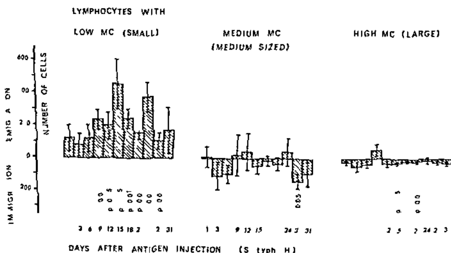


Fig 6

S typhi H

The percentage of small lymphocytes was decreased during the first 12 days after antigen injection. A corresponding increase in medium sized and large lymphocytes occurred, the increase being maximal 9 and 12 days after injection ($p < 0.001$). The changes were identical in thymic vein and carotid artery blood. From day 15 and onwards the percentage of different categories of blood lymphocytes had returned to the same level as in the normal animals (Fig 1)

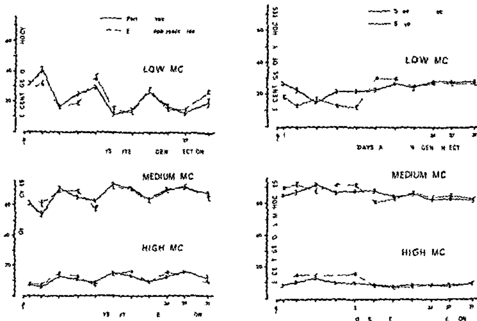


Fig 7

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph from guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi H* antigen The arrow indicates injection of antigen Mean ±S E

During the first 12 days after administration of the typhus antigen the number of small blood lymphocytes (with low MC) was low but increased at 15 days The number of medium sized and large cells rose monophasically with a maximum after 9 days No cyclical variation in the number of different blood lymphocytes like that in the animals given the pertussis or coli antigen was found (Fig 2)

A significant veno arterial difference in number of small lymphocytes in the thymic vessels was observed An import of large lymphocytes into the thymus was demonstrated after antigen injection (Fig 6)

In the thoracic duct lymph as in the blood the percentage of small lymphocytes was slightly decreased and that of medium sized and large lymphocytes correspondingly increased during the first 12 days after antigen injection (Fig 7)

DISCUSSION

The pertussis vaccine and the lipopolysaccharide from *E coli* caused the same cyclical variation in composition of the lymphocyte population in both blood and lymph As regards the percentage of different categories of lymphocytes in blood and lymph the similarity was striking but also the number of lymphocytes per mm³ of blood showed the same cyclicity despite a relatively great variation between the total lympho

cyte content of the blood in individual animals In both blood and lymph the incidence of small lymphocytes was maximal about 3 12 and 21 days after antigen injection when the numbers of medium sized and large lymphocytes were minimal Minimal values for small lymphocytes were found about 6 and 15 days after injection when the values for medium sized and large lymphocytes were maximal The *S typhi H* antigen and the sheep erythrocytes did not produce the same cyclical variation in the different categories of lymphocytes as did the pertussis and coli antigens

The cause of the varying influence of the different antigens on the circulating lymphocyte populations is not known The pertussis and the coli lipopolysaccharide antigens both of which caused the cyclical variation have several characteristics in common They both contain a lipopolysaccharide antigen are stable remain in the body for a long time induce production of 19S antibodies and usually no 7S antibodies A cyclical variation recalling that described in this paper has been reported in the number of plaque-forming cells in the spleen after immunization with the lipopolysaccharide from *E coli* (*Britton & Möller* 1966) The number of such cells is found to be correlated to the 19S antibody titres (*Wigzell et al* 1966) The maximal number of splenic immunocytes was recorded 6 15 and 24 days after antigen injection corresponding to our maxima for medium sized and large lymphocytes However the cyclical variation need not necessarily be caused by the antigenic properties but *e g* by some toxic property of the pertussis vaccine and the coli antigen

The coli lipopolysaccharide antigen caused a significant export of medium sized lymphocytes from the thymus after 15 and 18 days (Fig 4) A slight export at this time was also found in the animals injected with pertussis vaccine No such export of medium sized lymphocytes was observed in the animals given the other two antigens nor was it found in earlier experiments with normal and steroid treated guinea pigs Concurrently with this export the number of such cells in the blood was high

The sheep erythrocytes and the *S typhi H* antigen caused a monophasic increase in the percentage of medium sized and large lymphocytes in blood and lymph with a maximum after 6–12 days This increase was most pronounced as regards the *S typhi H* antigen coinciding with the appearance of agglutinating antibodies against the H antigen (*Ernstrom* 1965) It seems possible that these medium sized and large lymphocytes partake in the immune response perhaps as immunocytes

Injection of pertussis vaccine resulted in a large number of small lymphocytes in the blood during the early period after injection The animals injected with the lipopolysaccharide from *E coli* and the *S typhi H* antigen had low numbers of small lymphocytes in the blood during this period At this time the thymic export of small lympho

cytes was relatively low. This early deficiency of small lymphocytes may have been due to a stimulated secretion of adrenal steroids since previous results have shown that exogenous steroids decrease the thymic export of small lymphocytes (*Ernstrom & Larsson* 1967b). An activation of the adrenals caused by the antigen injection is especially plausible in the animals given the coli lipopolysaccharide, which was obviously toxic in the dose administered as some animals were ill on the first day after the injection and a few of them died.

The thymic export of small lymphocytes varied after the antigen injections. As a rule, a large export was accompanied by a high number of small lymphocytes in the blood and a small export by a low number. This was most obvious after injection of the lipopolysaccharide from *E. coli* which caused an interrupted thymic export of small lymphocytes after 15 and 24 days, coinciding with a minimal number of such cells in the blood.

After administration of the different antigens an import of medium sized and large lymphocytes into the thymus was observed at certain intervals after injection. Such an import has recently been demonstrated during the regenerative phase after steroid induced thymic involution (*Ernstrom & Larsson* 1967b). This implies that the present finding may not necessarily be caused directly by the antigens or by some immune mechanism during the primary antibody response but by adrenal activation and a steroid influence on the thymus.

In all four experiments with the different antigens the granulocytes behaved similarly. Thus pronounced but temporary granulocytosis was seen 1 day after injection. About normal numbers were registered after 3 and 6 days, but after 9–12 and 21–24 days the number of granulocytes in the blood was again raised. The initial granulocytosis observed 24 hours after antigen injection may have been due to adrenal activation, as exogenous steroids produce pronounced granulocytosis within 6 hours (*Ernstrom & Larsson* 1967b). A tendency to irregular cyclical variation in granulocytes was thus observed.

The present study has demonstrated that two bacterial lipopolysaccharide antigens (from *B. pertussis* and *E. coli*) cause an almost identical cyclical variation in the circulating lymphocytes of different categories in blood and lymph, and that two other antigens (sheep erythrocytes and *S. typhi H*) do not cause such a cyclical variation.

SUMMARY

Young adult guinea pigs were given a single intraperitoneal injection of one of the following antigens: pertussis vaccine, a lipopolysaccharide from *E. coli*, sheep erythrocytes, *S. typhi H*. The lymphocyte populations of carotid artery and thymic vein blood and of thoracic duct lymph were studied at different intervals after antigen injection. The lymphocytes were divided into subpopulations classified by the mito

chondrial content of the cells (correlated to the size of the lymphocytes) The difference between the number of lymphocytes per mm^3 of blood from the carotid artery and from the thymic vein indicates a thymic import or export of blood lymphocytes The following observations were made

1 A similar cyclical variation in composition of the circulating lymphocyte populations in blood as well as in lymph was caused by the pertussis vaccine and by the lipopolysaccharide from *E coli* A maximal percentage and number of small lymphocytes coincided with a minimal incidence and number of medium sized and large lymphocytes in blood and lymph about *3 12 and 21 days after antigen injection* A minimal percentage and number of small lymphocytes and maximal of medium sized and large lymphocytes occurred after 6 15 and 27 days

2 The *S typhi H* antigen and the sheep erythrocytes did not cause the same cyclical variation in the subpopulations of the lymphocytes as did the two lipopolysaccharide antigens The H antigen produced a monophasic increase in the incidence and number of circulating medium sized and large lymphocytes in blood and lymph with a maximum 9 days after antigen injection The sheep erythrocytes also *tended to induce a monophasic increase in medium sized and large* lymphocytes

3 An export of small lymphocytes from the thymus was found in the animals injected with the different antigens as in normal animals As a rule a large thymic export was accompanied by a high number of small lymphocytes whereas a small export was accompanied by a low number An import of medium sized and large lymphocytes into the thymus was observed without any obvious differences between the animals injected with the different antigens Part of the effects observed may have been mediated by an increased secretion of thymolytic steroids from the adrenals in the antigen treated animals

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IRRADIATION INDUCED ASYMMETRY OF THE THYMUS IN MICE

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The central role of thymus in the development of radiation induced lymphoid tumours in mice is well established (1). Spontaneous as well as virus induced thymus lymphoma may be preceded by a phase of lymphocyte depletion (2–5). With the intention to elucidate more precisely these problems concerning the radiation induced lymphoma the histological changes in the thymus after total body x-ray exposure were systematically studied and analysed from the time of irradiation up to the development of thymus lymphoma.

MATERIAL AND METHODS

The experiments were conducted on mice of the C3H strain since the slow development of lymphoma in this strain makes it easier to follow the development of the tumours. 293 males and 297 females 25 ± 2 days old were irradiated with 500 R administered in 4 equal fractions at intervals of 4 days (6). Following the 1st irradiation the animals were killed in groups of from 17 to 53 animals during the first month every second to every third day, during the second month every 10th day and thereafter monthly from the 3rd–7th month. 84 males and 78 females served as untreated control animals. The thymus was removed by careful dissection with intact capsel. The two lobes were separated and weighed immediately on a torsion balance with the accuracy of ± 0.05 mg. The lobes were fixed in Stieve's fluid for histological examination.

RESULTS AND DISCUSSION

The absolute weight difference of the lobes of the thymus decreased in the control animals with increasing age and decreasing total weight of the thymus (Fig. 1). *In the experimental animals however a successively more accentuated weight difference of the lobes of the thymus occurred in the beginning (weight asymmetry).* The maximum difference appeared between the 23rd and 27th day after the last irradiation when the total weight of the thymus once again was increasing. During this period the weight difference ranged between 5.1–10 mg in 29 per cent of the animals, between 10.1–15 mg in 13 per cent and above 15 mg in 8 per cent. In the control group the weight difference was under 5.1 mg in 95 per cent of the animals. When the total weight of the thymus had

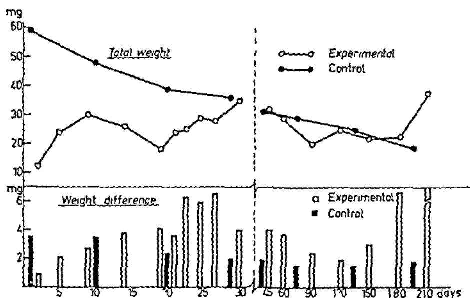


Fig 1

Mean total weight and mean weight difference of the lobes of thymus in C3H mice after 500 R of total body irradiation divided into four equal doses given every 5th day

returned to normal in the experimental animals after the first month the weight difference decreased only to increase again after 5–6 months with the appearance of an initially unilateral lymphoma.

In the period immediately following the last irradiation the thymus followed histologically the earlier known pattern (7). On the second day the cortex was thin with a diffuse border toward the medulla. The number of lymphocytes had decreased and reticular cells and immature lymphoid cells dominated the picture.

On the 5th day a broader more dense partially regenerated cortex was seen and on the 9th day the first regeneration seemed to be completed. These changes appeared regularly and symmetrically in both lobes. After the 14th day the cortex became again thinner and sparse in cells and showed a diffuse border against the medulla. The number of lymphocytes was reduced and once again reticular cells became more apparent. This secondary depletion was in general symmetrically distributed in both lobes. From the 19th day the histological picture was generally more uniform within the individual lobes at the same time as the differences between the lobes of the same thymus were more marked (*early histological asymmetry*) (Fig 2).

By now the heavier lobe had recovered from the second depletion showing a cortex in regeneration or a more normal cortex. In contrast the lighter lobe was remained at the same time depleted. This depletion was occasionally further accentuated with almost complete disappearance of lymphocytes as a result (Fig 2). At this stage the cortex was



Fig 2
Transverse section of thymus lobes of C3H mice 25 days after irradiation
Haematoxylin and eosin

dominated by scattered reticular cells and the picture was similar to that obtained 2–3 days after irradiation with 1 × 550 R the cell density in the medulla being greater than that in the cortex This second depletion was followed by a phase of regeneration which in the main agreed with the first to occur after irradiation Immature lymphoid cells with a finer chromatin structure and basophilic cytoplasm formed successively a broad mitosis rich cortex surrounding small diffusely bordered medullary islands Further development resulted in an apparently normal lobe of increased size and weight In certain cases an abnormally broad cortex was seen in which medullary islands were absent or very small

Kaplan & Brown (8) observed a similar picture of the thymus in C57Bl mice it appeared during the regenerative phase immature cells were present together with a high mitotic activity which was interpreted as an apparent impairment of differentiation The picture was similar to that of an early lymphoid tumour *Wasi & Block* (9) also reported the finding of a temporary lymphocytic depletion in the thymus about 3 weeks after irradiation of C57Bl mice

Once the total weight of the thymus had returned to normal after the first month the number of cases of asymmetry reduced although such asymmetry was seen throughout the rest of the experimental period (*late histological asymmetry*) the pictures thus exhibited were not divergent from that of the early histological asymmetry described above In addition after 5–7 months mice were seen in which the immature lymphoid cell populations in the lighter lobe showed an increased number of mitosis and an increased number of hyperchromatic and pyknotic nuclei during the regenerative phase In other mice these

changes were more accentuated coincident with the affected lobe now being heavier than normal and obviously the seat of a lymphoma The transition from a regenerative phase to a lymphoma was thus very gradual In addition to this cases of advanced bilateral lymphoma were also seen

Similar late asymmetrical depletion has previously been described as a precursory stage of spontaneous lymphoma in the thymus of 5–8 months old AKR mice (3) Asymmetrical depletion may also precede the development of virus induced thymus lymphoma in the mice (5)

In order to study more closely the further development of the asymmetrical thymus lobes biopsies were done on animals 23–29 days after the last irradiation Under anaesthesia the thymus was exposed and a small piece of each lobe was carefully excised for histological examination whereafter the operation site was closed again In 4 cases in which the biopsy material showed an accentuated lymphocyte depletion in one lobe and relatively normal condition in the other a reversed condition between the lobes was found at the time of sacrifice and autopsy 20 40 and 50 days later The lobes presenting depletion in the biopsy material showed now relatively normal cell picture in three cases and immature regeneration in the fourth case In contrast the lobes presenting normal conditions in the biopsy material now showed in three cases depletion and in one case regeneration Serial sections of 10 lobes with accentuated depletion or regeneration showed in the majority of cases a histological picture that was relatively uniform throughout the entire lobe

Since no degenerated lobes were seen it is probable that such alternating depletion and regeneration reflect different phases of a reversible reaction which apparently starts with an emptying of the cortex of lymphoid cells and then develops into a variable reaction pattern with successive regeneration and normalization alternating between the lobes A phase displacement between the lobes may here mean that they have become unsynchronous which in turn is a conceivable explanation of the asymmetry which has arisen

In order to study the possible connection between this asymmetrical thymus picture and the x irradiation animals in another experiment were irradiated with 550 R administered as a single dose As expected fewer lymphomas were obtained (6) but also fewer and less marked asymmetries than with fractionated irradiation (in preparation) On the other hand *Kaplan & Brown* (8) found essentially the same reaction picture in the thymus following single dose or fractionated doses (same total dose 476 R) administered to C57Bl mice

Since thigh shielding prevents the development of a secondary weight reduction in the thymus (8) and inhibits the occurrence of radiation induced lymphoma in the mouse (10) an experiment was conducted in order to ascertain the effect if any on the occurrence of the asymmetrical thymus picture The left thigh of 24 animals was shielded during the irradiation (550 R in 4 fractions as above) When these

animals were autopsied on the 25th day the mean weight difference between the lobes of the thymus was 5 5 per cent of the total weight In the untreated control animals the corresponding value was 6 3 per cent and in unshielded irradiated animals 27 5 per cent

A detailed report will be presented elsewhere

SUMMARY

Histological investigations of the histogenesis of radiation induced leukaemia in the C3H mouse have shown that an asymmetry of the lobes of the thymus as regards weight and histological pictures begins to appear during a secondary regenerative phase following irradiation This asymmetry is observable for several months and seems to be correlated with the occurrence of unilateral lymphomas

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the cytologi al estimate of tumour type and degree of differentiation Surgical or autopsy material is available for the histopathological follow up of 304 patients Malignant tumour was cytologically diagnosed in 373 samples (40 9 per cent) and 50 samples (5 5 per cent) were considered suspicious of malignancy These 423 biopsy samples represent 333 patients As regards 209 of these patients material for histo pathological follow up is available Out of the other 124 patients 31 have died the major clinical diagnosis in all cases being cancer but autopsy was not performed The remaining 93 patients are still alive and are followed clinically

The number of aspiration biopsies has steadily increased but because of the shorter observation period fewer histopathological comparisons can be made on the 1965 material The number of patients per year with a diagnosis of malignancy is given in Fig 1

TABLE 1

Localization of the Tumours in 209 Patients with Histological Follow Up

Site (lobe)	Histological diagnosis: Epidermoid carcinoma	Adeno carcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	Other	Total number of patients
Right upper	35	15	8	6	–	4	68 (33%)
Right middle	3	–	–	2	–	–	5 (2%)
Right lower	14	7	4	5	4	–	34 (16%)
Left upper	33	14	4	11	3	1	66 (32%)
Left lower	13	7	4	1	4	2	31 (15%)
Mediastinum	–	–	–	–	4	1	5 (2%)
Total	98	43	20	25	15	8	209 (100%)

The sites of the infiltrates sampled are shown in Table 1 Tumours were located in all lobes of the lungs and perihilar as well as peripheral tumours were diagnosed The approximate diameter of the lesions was measured directly on the surgical or autopsy specimens in most instances If a long time elapsed between aspiration biopsy and operation or autopsy or if other considerations made direct measurement unsatisfactory the diameter was estimated on the basis of radiograms with due allowance for the geometric distortion caused by X ray divergence In the few in stances of poorly demarcated lesions measurement was not attempted The size distribution is illustrated in Fig 2

Among the 209 patients from whom histopathological material was available 46 were women and 163 men Their ages ranged from 23 to 80 years (Fig 3)

The entire histopathological material has been re examined by one obser er with a view to obtaining uniformity in evaluation The sections were stained a ording to van Gieson s method and with haematoxylin eosin

RESULTS

A Tumour Incidence

A cytological diagnosis of malignant tumour was established in 192 patients This diagnosis could be verified on the basis of the histo pathological examination of surgical or autopsy material obtained from 188 patients (Table 2)

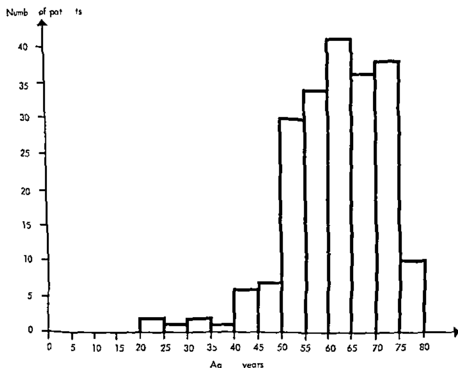


Fig 3
Age distribution of the patients

TABLE 2
Evaluation of Lung Tumours in 209 patients with Histological Follow Up on Surgical or Autopsy Specimens

Cytological diagnosis	Histological diagnosis						
	Malignant tumour	Tuberculosis	Non-specific inflammation	Radiation pneumonitis	Hamartoma	Thymoma	Total
Malignant cells	188	2	1	1	–	–	192
Malignancy suspected	13	2	–	–	1	1	17
Total	201	4	1	1	1	1	209

Thus in the case of four patients (2.1 per cent) a false positive diagnosis of malignancy had been established In two cases tuberculous changes observed in the cytological material were misinterpreted as malignant tumour In one patient earlier irradiated for mammary carcinoma a focal infiltration in the lung was diagnosed as an adeno-

carcinoma. Examination of the histological material, however, revealed no signs of malignancy: the lesion represented chronic inflammation and fibrosing, apparently radiation induced. In a fourth patient a focal infiltration was diagnosed cytologically as a malignant tumour but after histological examination the lesion was found to be an interstitial pneumonia, probably resulting from aspiration.

Among the patients in whom malignancy was suspected on the basis of the cytological findings (17 included in the histopathological follow-up) 13 were found to have malignant tumours. In two patients localized tuberculous changes were observed and one had a cellular chondromatous hamartoma. In one patient a cytological sample aspirated from the mediastinum aroused the suspicion of a manifestation of malignant lymphoma or small cell cancer of oat cell type that turned out to be a *thymoma*.

Histopathological follow-up of the 95 patients who on the basis of the cytological findings had not presented evidence or suspicion of malignancy revealed that malignant tumours were manifested in 15 cases. These 15 patients represent the false negative diagnoses. Material inadequate for cytological evaluation had been obtained from eight patients. Only inflammatory changes were seen in material aspirated *from the other seven patients* (Table 3).

In the total follow-up material comprising 304 patients, 215 malignant tumours were found. Cytological examination disclosed 87 per cent of these and gave rise to a suspicion of malignancy in a further six per cent. In 15 patients (seven per cent) with malignant tumours a cytological diagnosis was not obtained.

TABLE 3

Evaluation of 15 Patients with False Negative Diagnoses

Cytological diagnosis	Histological diagnosis: Epidermoid carcinoma	Adeno carcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	Total number of patients
None	2	1	–	2	3	8
Inflammation (not specified)	3	–	1	–	2	6
Tuberculosis		–	–	–	1	1
Total	5	1	1	2	6	15

B. *Type of Tumour*

Cytological typing was attempted in a material comprising 282 patients with a cytological diagnosis of malignancy. The steady increase

in numbers of biopsies has made it possible to gain the experience required if the desire on the part of clinicians is to be fulfilled and more specific diagnoses are to be established. The relative number of cytologically typed tumours have increased from about 75 per cent in 1963 to 93 per cent (1964) and 98 per cent (1965). Histopathological material from 184 patients was available for a comparison with the type diagnosis made on the corresponding cytological sample.

TABLE 4
Cytological Results According to Histological Type of Tumour

Cytological diagnosis	Histological diagnosis: Epidermoid carcinoma	Adeno carcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	Other	Total number of patients
Epidermoid carcinoma	76 (88%)	10	4	3	–	1	94
Adenocarcinoma	4	24 (60%)	1	4	1	–	34
Oat-cell carcinoma	1	2	12 (67%)	–	–	–	15
Undifferentiated carcinoma	3	4	1	17 (71%)	–	1	26
Metastasis	2	–	–	–	12 (92%)	1	15
Total number of patients	86	40	18	24	13	3	184

A comparison between the cytological and histopathological type diagnoses is given in Table 4. Cytological typing was correct in 77 per cent of the patients. Optimal agreement (88 per cent) was obtained in the 86 cases of epidermoid tumours. As regards the somewhat smaller groups of anaplastic tumours and oat cell tumours the original cytological typing was correct in 71 per cent and 67 per cent of the cases respectively. Adenocarcinoma was most difficult to classify correctly—only 60 per cent were correctly recognized in the cytological samples. In fact 25 per cent (10 tumours) of the adenocarcinomas were cytologically typed as epidermoid tumours. Twelve of the 13 metastatic tumours were correctly recognized cytologically.

C. *Degree of Tumour Differentiation*

Cytological material sufficient for an evaluation of the degree of tumour differentiation was obtained from 194 patients. Histopathological material to be used for a comparison was available from 122 of these patients. Any special interest in this aspect of diagnostics has not

been taken until late 1964 which may account for the relatively few cases that are available for a comparison

The degree of differentiation was denoted as high moderate low or anaplastic If a mixed description was used for example moderately to highly differentiated the lower degree has been chosen for the purposes of this comparison

A comparison between degrees of differentiation in the cytological and histopathological material is given in Table 5 There was good agreement in 78 per cent of the patients The degree of differentiation was often underestimated in the cytological material obtained from moderately differentiated tumours

TABLE 5

Cytological Results According to Degree of Tumour Differentiation

Cytological diagnosis Differentiation	Histological diagnosis Degree of differentiation Anaplastic	Low	Moderate	High	Total
Anaplastic	18 (95%)	1	–	–	19 (16%)
Low	1	52 (88%)	14	2	69 (58%)
Moderate	–	6	20 (59%)	2	28 (23%)
High	–	–	–	4 (50%)	4 (3%)
Total	19 (16%)	59 (49%)	34 (28%)	8 (7%)	120 (100%)

DISCUSSION

Tumours account for about 45 per cent of all our aspiration biopsy material (*Dahlgren & Nordenström* 1966) Since an exact diagnosis of lung infiltrations can rarely be achieved on the basis of radiograms alone complementary methods are necessary to sort out the ever increasing group of patients with radiological evidence of lung infiltration The poor prognosis associated with late detection of bronchial cancer is a spur to obtain a diagnosis as early as possible There is every reason to have rapid and simple methods developed with a view to obtaining a diagnosis as exact as possible in order to have the adequate therapy instituted Aspiration biopsy guided by television X ray permits us to reach even small and relatively inaccessible tumours

The histopathologically verified tumours in patients in this series fit in with the age and sex pattern seen in larger series of bronchial carcinomata As has been the case in other published series (*Liebow* 1952 *Spencer* 1962) the upper lobes predominate as tumour sites There is no distinct difference between the right and left sides Epidermoid tumours and the undifferentiated cancers predominate in the upper lobes

With the technique used here a cytological diagnosis can be obtained in from 85 to 90 per cent of cases of malignant intrathoracic tumours which can be detected radiologically. This result is to be seen against its background—aspiration biopsy was carried out on all types of radiologically visible lung infiltrations, not solely on those suspected of malignancy.

A false positive diagnosis was made in about two per cent of the cases. Several factors have contributed to these diagnostic errors. Tuberculosis and bronchiectases particularly, but also other chronic processes exert an irritating effect on epithelial cells, thus producing a wide pattern of changes dependent upon the duration and degree of the irritation (*Grunze* 1955, *Koss* 1961). Degeneration or activation of epithelial cells can lead to heavy nuclear hyperchromasia with large nucleoli and vacuoles. The nuclear cytoplasm ratio can also be influenced. In routinely stained cytological materials, these changes resemble those seen in malignant epithelial tumours. Only the degree of these morphological changes may distinguish an inflammatory cell reaction from a neoplastic change. Increased cell activity in inflammatory reactions can also result in a significant degree of basal cell hyperplasia. In some instances the degree and relative abundance of inflammatory epithelial changes can dominate the cytological material obtained by aspiration biopsy to such an extent that they may simulate the appearance of a malignant tumour. In the present series tuberculosis in two patients and a non specific chronic inflammation in a third together with the accompanying inflammatory changes in epithelial cells are probably the source of the erroneous diagnoses of malignancy.

Another source of error is that cells profoundly altered by radiation can be misinterpreted as malignant cells. *Graham* (1963) as well as others have described such cellular changes after radiation therapy. She observed that radiation induced enlargement of the cells and their nuclei with vacuolization particularly of the cytoplasm but also within the nuclei. Bizarre and sometimes multinucleated cells also appeared. These changes which may persist long after radiation therapy has been terminated can closely resemble the ones seen in cancer cells. The risk of diagnostic errors is always present if the cytologist is not informed about radiation of the region from which the sample was obtained. One of the patients in this series had received both pre and postoperative radiation therapy for mammary cancer. During a routine radiological check a focal infiltration was detected in the right upper lobe. Two attempts at aspiration biopsy were made: the first was cytologically inadequate and the second gave fairly large polymorphous cells with distinct nucleoli. These were assumed to originate from an adenocarcinoma. When the resected segment was examined histopathologically it became apparent that the focal changes were radiation induced and any tumour tissue was not detected.

Russell et al (1963) have also emphasized the risk of contamination between samples as a source of false positive diagnoses There is reason to suspect that cross contamination occurred on one occasion in the present series

Among the cytological samples which wrongly were suspected of malignancy later histopathological examination demonstrated the presence of a hamartoma More recently it has become possible apparently to diagnose hamartomas cytologically (*Dahlgren* 1966) Re examination of the original cytological sample from the patient mentioned above did in fact reveal typical hamartoma features In this particular instance inexperience in the cytological appearance of this type of lesion resulted in the establishment of a wrong diagnosis

With current methods of examination it is unlikely that false positive diagnoses can be wholly avoided This applies of course to all fields of exfoliative cytology (*Koss* 1961 *Russell et al* 1963) With improved methods the level of false positive diagnoses can probably be reduced still further

Malignant tumours were not diagnosed in 15 patients The principal cause of these false negative diagnoses seems to be that inadequate material had been obtained by aspiration Either the tumour has been missed by the needle or else the tip of the needle may have entered devitalized portions of the tumour Nearly all tumours are surrounded by a zone of chronic inflammation If material is aspirated from this zone it is deceptively easy to be satisfied with a cytological diagnosis of inflammation

The technique of aspiration biopsy gives the possibility of obtaining representative cells from typical and viable portions of a tumour and thereby facilitates the identification of tumour cells There was good correlation (77 per cent) between cytological tumour type and histopathological tumour type It is remarkable then that the correlation was only 60 per cent in the case of adenocarcinomas Some 25 per cent of the adenocarcinomas were wrongly typed as epidermoid tumours It was predominantly the poorly differentiated adenocarcinomas which accounted for the errors Difficulties involved in a correct cytological recognition of adenocarcinomas has also been experienced in extensive series of exfoliative sputum cytology (*Russell et al* 1963) One reason for these difficulties may be that the staining methods used are most suitable for the recognition of epidermoid tumours Since the poorly differentiated adenocarcinomas usually form only scanty mucus special stains for mucus are rarely a diagnostic aid

Any publications are not available as regards figures obtained in evaluations of degrees of tumour differentiation and comparable with our findings in similar attempts A comparison between the degree of differentiation judged on the basis of cytological samples and findings in histopathological specimens gave agreement in about 78 per cent of the cases There was a tendency to have the cytological degree of

differentiation placed at a rather too low level A possible explanation of this may be that the lowest differentiated cells have a lower degree of adhesion and come to be overrepresented in aspiration biopsy samples (*Coman* 1944) Another source of error involved in a comparison of this type is the occasionally long interval between the performance of aspiration biopsy and the time when material for histopathological examination is obtained During this interval a tumour can assume a lower but hardly a higher degree of differentiation

The degree and direction of tumour differentiation can vary in different parts of a tumour For this reason the cytological diagnosis made on isolated cells can never be as conclusive as the histopathological diagnosis based on examination of relatively large areas of a tumour

The results obtained in these patients demonstrate that it will be possible in numerous cases to decide on the basis of cytological samples obtained by aspiration from radiologically evident lung lesions whether or not the lesion is malignant and also the type and degree of tumour differentiation Considering the greater range of therapeutic alternatives in cases of lung tumours these aspects are of importance

Further developments in this field of diagnostics are imperative Even if the results to a large extent are dependent upon the personal experience of the cyto pathologist more subtle methods of fixation and staining can be expected to bring about improvement

SUMMARY

Fine neddle aspiration biopsies with the aid of television fluoroscopy were undertaken with a view to obtaining the cytological diagnosis of intrathoracic lesions During a three year period 912 aspiration biopsies were performed on 667 patients Malignant tumours or suspicion of malignancy were cytologically diagnosed in 333 patients To evaluate the diagnostic reliability of this method in cases of malignant intrathoracic tumours findings in 304 patients were compared with the histopathological material obtain d at operation or autopsy The results obtained demonstrate that malignan intrathoracic tumours could be correctly diagnosed cytologically in bout 87 per cent of the cases in a further six per cent the tumour w[illegible] cytologically diagnosed as malignancy suspected In about 77 per cent of the patients it was also possible to determine corr[illegible]ly the tumour type and the degree of tumour differentiation

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EXPERIMENTAL STUDIES ON BOVINE TICK BORNE FEVER

2 *Differences in Virulence of Strains in Cattle and Sheep*

By

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Received 20 xii 66

Foggie (1) reported that the virulence of strains of ovine tick borne fever agent he had studied could differ in sheep Evidence which points in the same direction is to be found in the wide divergences in description by various authors of the severity of tick borne fever in sheep (7 9 13)

In the preceding report of this series an account was given of the isolation of 10 strains of tick borne fever agent from bovine field cases and one strain from unfed nymphs of *Ixodes ricinus* (16) Some of the strains have been studied each of them in several experimental cattle and sheep The present paper describes the differences observed in their virulence in the respective hosts

MATERIAL AND METHODS

The material and methods employed in this study have been mainly presented in the preceding paper of the series (16) Some additional information is given below

Field cases from which the agent strains were isolated and the conditions The geographical origin of each isolate has been presented previously (16) Table 1 provides some information on the field cases concerned The findings are unsatisfactory for comparative purposes as the time at which each cow was examined the blood samples were taken and treatment administered varied in relation to the beginning of the reactions

The unfed nymphs of *I ricinus* from which J-Puke strain was isolated (15) were collected in spring 1965 from a farm pasture on which both ... cows had been simultaneously attacked by tick borne fever in summer 1964 The cows had exhibited depression diminished appetite and a substantial fall in the milk yield The maximum temperatures recorded were 41 4 and 41 5 C and the percentages of granulocytes displaying tick borne fever bodies 38 and 41 respectively Both cows had coughed and one had had diarrhoea The data should be compared with the reactions J-Puke strain had caused in experimental cattle On this farm sheep had been kept continuously and grazed on the same pasture as cattle

A brief account of some of the present findings was given at the IX International Congress for Microbiology in Moscow but has been published only as an abstract (14)

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TABLE

Tick Borne Fever Reactions in 10 Lactating Cows in the

Strain isolated	Cow had been bought in recently	Cow had been reared at home	Age of cow Y	Interval from noticed start of illness to veterinary call days	Highest recorded temp C	Proportional fall in milk yield§
J34–64	×		3	0	41 2	total
Ju3–64		×	10	2	40 9	1/2
J48–64	×		2	1	41 1	1/2
J53–64	×		4	1	40 0	total
J1–65	×		2	1	41 5	total
K1–65	×		4	0	42 0	1/3
K20–65		×	2	1	41 2	1/2
T1–65	×		7	0	41 6	1/3
Jo5–65		×	3	1	41 ?	1/?
Jo6–65		×	5	1	40 9	1/2

n i = no information
n f r = not fully restored

After discovery of the high virulence of K20–65 strain and a recently isolated K9–66 strain in sheep further information was obtained on the conditions on the farms of their origin No sheep had been kept on these farms or on neighbouring farms for many years

Serial inoculations by heterologous strains In view of an extensive immunological heterogeneity among the strains to be described in the third paper of the series (17) many animals reacted to serial inoculation of several heterologous strains These reactions to heterologous strains in animals with previous experience of tick borne fever are partly taken into account in describing the differences in virulence of strains This refers especially to cases in which later reactions are more severe than the earlier ones

Criteria of virulence The following criteria were used 1 Percentage of granulocytes displaying tick borne fever bodies and the period during which the bodies are detected 2 Degree and duration of the temperaturee reaction and 3 Length of the incubation period

RESULTS

Degree and Duration of the Temperature Reaction

All the isolated strains invariably caused a temperature reaction when inoculated into cattle with no previous experience of tick borne fever Sequential inoculations with heterologous strains also often induced a temperature reaction Fig 1 indicates the duration of reactions by different strains both in cattle with and without previous experience of the disease No definite differences between the strains emerge from these comparisons In regard to many strains the experiments carried out are too scanty to allow of drawing conclusions However the impression was gained that as concerns the more extensively studied strains K20 65 tended to cause a longer fever reaction than J34 64 In Table 2 the strains are compared in respect of the highest measured temperatures they caused in older cattle without earlier experience of

1

Field from which Agent Strains Were Isolated 1964–1965

Depression§	Anorexia	Percentage of granulocytes showing bodies	Treatment	Restoration of previous milk production in days	Subsidence of fever in days
X	X	42	Tetracycline	1	1
X		20	Tetracycline	n r	n r
X		37	Tetracycline	n f r	2
		24	Tetracycline	7	1
X	X	6	Tetracycline	n f r	1
X	X	38	Sulphamezathine	7	2
X	X	25	Sulphamezathine	5	1
X	X	35	Sulphamezathine	n r	1
X	X	5	Tetracycline	n r	n r
X	X	16	Tetracycline	n r	n r

= temperature not recorded by the farm people
§ = recorded before or at the time of the veterinary call

the disease On the average the J34 64 and K20 65 strains resulted in a fever clearly higher than that induced by the J Puke strain In the next paper of the series evidence will be presented that cross immunity between Jo5 65 strain and other tested strains appears to be very weak or absent The mean of the highest recorded temperatures caused by this strain in 11 cattle including calves and adults was 41 2 C or within the same range as the figures given for the J34 64 and J1 65 strains

TABLE 2

Highest Recorded Temperatures in Reactions in Cattle over 1 Year Old with no Previous Experience of Tick Borne Fever by Different Strains

	Strain of agent							
	J34–64	K20–65	J-Puke	K1–65	T1–65	J1–65	J48–64	Jo5–65
Number of reactions	12	4	4	1	1	1	1	1
Range C	40 7–41 8	40 7–41 3	39 7–41 2	41 6	40 8	41 7	40 4	40 9
Mean C	41 3	41 1	40 5					

Fig 2 contains data on duration of the temperature reaction in sheep It becomes apparent that J Puke strain is more virulent in this respect than are J34 64 and J1 65 strains It should be noted that although the K20 65 strain was tested only in sheep which had reacted earlier to other strains it matched J34 64 and J1 65 as regards duration of the induced fever No temperature reactions were caused by Jo5 65 Jo6 65 and K1 65 strains Jo5 65 strain was inoculated into two sheep and

STRAIN OF AGENT	NO TEMP BUT TICK-BORNE FEVER BODIES	DURATION OF FEVER PERIOD DAYS 1	2	3	4	5	6	7	SECOND TEMP REACTION
J Pu Ke					●●●●				
J 48 64				●					●
Jo 5-65			○○	○	○○○	●●○○		○	○
J 34 64		○○○	●●●●●●●○○○	●●●●●●	●●●	●●●●			●●●●
J 1-65			○○	●○○○○	●	○	○		●○○
J 53 64		○		○	○				
T 1 65	○○		○		●				
Ju 3 64				●					
Jo 6 65						○			○
K 1 65		○	○○	●○	○○				●○
K 20 65		○	○○	○○	●●	●●			●●

● ANIMAL WITH NO EARLIER EXPERIENCE OF TICK BORNE FEVER
○ ANIMAL PREVIOUSLY INFECTED WITH ONE OR MORE HETEROLOGOUS STRAINS

Fig 1

Period of elevated temperature in primary tick borne fever reactions caused by various strains in experimental cattle

Jo6 65 strain into one sheep with no earlier experience of the disease Additionally Jo5 65 strain was inoculated into 6 sheep which had earlier reacted to other strains K1 65 was inoculated into 3 sheep of the latter type On the average both J Puke and K20 65 strains induced temperatures which were definitely higher than those attributable to J34 64 and J1 65

Occurrence of Tick Borne Fever Bodies

Fig 3 makes a comparative examination of the strains in respect of the maximum percentage of detectably infected granulocytes they caused in cattle The lower virulence of J Puke strain is apparent All the 4 cattle inoculated with this strain were fully grown animals The maximum percentages recorded in them were only 2 2 4 and 10 respectively As regards the other strains K20 65 appeared to cause higher levels of infection than three strains which were studied more extensively Jo5 65 J34 64 and J1 65 If all the animals infected with Jo5 65 strain are considered as virtually fully susceptible then the highest

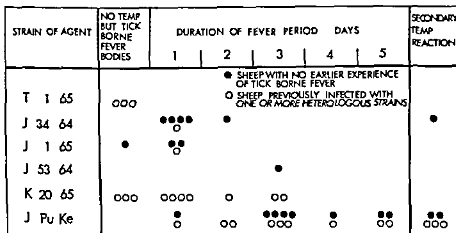


Fig 2
Period of elevated temperature in primary tick borne fever reactions caused by various strains in sheep

proportion of infected granulocytes caused by this strain tends to remain at a lower level than that attributable to J34 64 and J1 65 Attention is also drawn to the high percentage (72) of infected granulocytes caused by K1 65 strain in a heifer with no earlier experience of the disease the data should be compared with those for the respective field case in Table 1

Table 3 indicates that J PuKe strain is clearly inferior to other strains also as *regards the duration of the period in which tick borne* fever bodies were detected

TABLE 3

Period during which Tick borne Fever Bodies Were Detected in Cattle over 1 Year Old with no Earlier Experience of Tick Borne Fever by Different Strains

	Strain of agent							
	J34–64	K20 65	J–PuKe	K1–65	T1–65	J1 65	J48–64	Joo–65
Number of reactions	1?	4§	4	1	1	1	1	1
Rang days	3–8	4–5	1–2	6	3	7	4	7
Mean days	4 4	4 5	1 8					

and § = In 5 and 3 ttle re pe tively od es w not s ught bey nd the fourth or fifth da f their occurrence

Fig 4 presents the highest recorded levels of tick borne fever bodies in sheep by various strains The higher virulence of the K20 65 strain than J34 64 and J1 65 is more clearly evidenced by these data than by differences in the temperature reactions given in Fig 2 Note also that K20 65 strain seems to cause levels of tick borne fever bodies in sheep

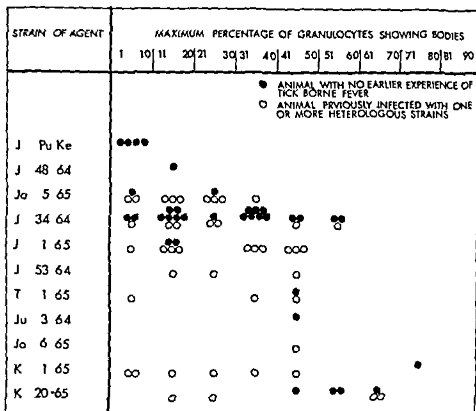


Fig 3

Highest percentage of granulocytes containing tick borne fever bodies recorded in experimental cattle reacting to inoculations by various strains
(Remarks) All the inoculated cattle without previous experience of tick borne fever reacted exhibiting tick borne fever bodies Negative inoculations into animals with previous experience of the disease are not included

which are at least as high as those in cattle (Fig 3) The maximum percentages of infected granulocytes caused by J Puke strain (up to 90 per cent) were higher than those resulting from any strain in cattle (K1 65 strain 72 per cent) T1 65 strain appears to be at least as virulent in sheep as J34 64 and J1 65 if measured by the level of infected granulocytes

The pattern displayed by different strains as regards the induced length of the period during which tick borne fever bodies were detected in sheep showed a correlation with that recognized for the maximum percentages of infected granulocytes

Sequential Reactions by Heterologous Strains

Differences between the strains described in the foregoing are substantiated by consideration of the sequential reactions to heterologous strains in individual animals The above comparisons suggested that

STRAIN OF AGENT	NO BODIES	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90
Jo 5 65	●●									
Jo 6 65	●									
T 1 65		○○		○						
J 34 64		●● ○	●●	●						
J 1 65		●●	●	○						
J 53 64				●						
K 20 65				○○○	○	○○		○○○	○	
J Pu Ke					●● ○○	● ○	○○	●● ○○	● ○	●● ○

MAXIMUM PERCENTAGE OF GRANULOCYTES SHOWING BODIES

● SHEEP WITH NO EARLIER EXPERIENCE OF TICK BORNE FEVER

○ SHEEP PREVIOUSLY INFECTED WITH ONE OR MORE HETEROLOGOUS STRAINS

Fig 4

Highest percentage of granulocytes containing tick borne fever bodies recorded in experimental sheep reacting to inoculations by various strains Negative inoculations into sheep without earlier experience of tick borne fever are included
(Remark) Negative inoculations into sheep with previous experience of the disease are not includ d

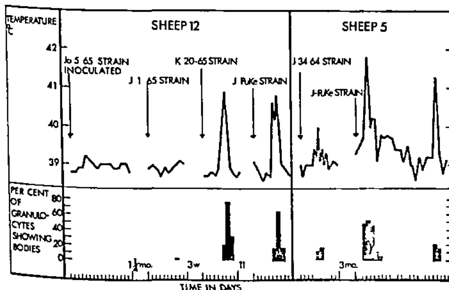


Fig 5

Comparison of tick borne fever reaction caused by l n ulations of heterologous strains (5 in all) in she p

with regard to the extent of the proportion of infected granulocytes produced in cattle four agent strains seemed to be arranged in the following order of virulence K20 65 (most virulent) J34 64 Jo5 65 and J Puke The results compiled in Table 4 further support this contention Although K20 65 strain was inoculated into three calves subsequent to one or two of the other strains nevertheless it induced higher levels of infected granulocytes The inferiority of J Puke strain to Jo5 65 and J34 64 strains was also demonstrated

TABLE 4

Comparison of 4 Agent Strains highest Percentages of Detectably Infected Granulocytes Induced in 6 Cattle Serially Inoculated by Two or Three of the Strains

Animal	Strains causing reaction their order of inoculation and the highest recorded percentage of infected granulocytes				
	1 strain	2 strain	3 strain	4 strain	5 strain
Calf 3	J34-64 (30%)	×	Jo5-65 (30%)	×	K20-65 (63%)
Calf 4	×	Jo5-65 (40%)	×	×	K20-65 (68%)
Calf 23	×	×	Jo5-65 (16%)	×	K20 65 (40%)
Cow 22	J-Puke (4%)	Jo5-65 (16%)			
Calf 26	Jo5-65 (28%)	J34 64 (52%)			
Cow 29	J-Puke (2%)	J34-64 (28%)			

× = reaction attributable to a strain not included in the comparison concerned

Differences between some strains in respect to their virulence in sheep are indicated in Fig 5 The greater virulence of J Puke and K20 65 than J34 64 and J1 65 strains is exemplified as is the non virulence of Jo5 65 strain in sheep (A strain K9 66 has been recently isolated from a bovine field case and found to resemble K20 65 in its virulence in sheep)

Incubation Period

The incubation period in cattle inoculated with blood obtained at about the height of the tick borne fever reaction ranged from 4 to 10 days (Two cases in which the incubation period was prolonged obviously by partial homologous immunity are not taken into account here) This refers to all the strains examined and includes inoculations with both fresh and stored blood No definite differences were observed in the incubation period between strains isolated from bovine field cases However J Puke strain tended to have a longer incubation period than the other strains this is apparent in the experiments described later in this paper

In sheep the incubation period varied from 2 to 9 days J Puke strain was the only strain capable of producing a clinical infection in two days On most occasions the incubation period with this strain was 2 to 3 days In two sheep inoculated with fresh blood containing K20 65

strain the incubation period was 3 days Even blood infected with K20 65 strain and stored at —74 C for 1 month once induced the appearance of the first tick borne fever bodies in three days With all other strains the incubation period was 4 days or more

In tests with the K20 65 strain it appeared that the incubation period can apparently be prolonged under certain interesting conditions

Four sheep were inoculated intravenousl with 1 ml fresh blood obtained from a sheep at the peak of reaction to K20–65 strain Three of the recipient sheep had experienced infection by J–Puke strain 1 –3 months earlier but one of them sheep 8 was at the time of inoculation recovering from the reaction caused by J–Puke strain It had experien ed the peak of reaction three days previously but even at the time of inoculation tick borne fever bodies were detectable in a few granulocytes The incubation period in two sheep was three days and in the third one in which it was suspected that the inoculati n had been effected subcutaneously by accident four days In sharp contra t with these the incubati n period in sheep 8 was 9 da s The reaction which developed in sheep 8 equalled that in other sheep in respect of the level of infected granulo tes The elevati n of temperature was slight in all the sheep but was hardly recognizable in sheep 8 When challenged about three weeks later sheep 8 proved to be solidly immune against K20–65 strain

It seemed reasonable to assume that infection by J Puke strain in sheep 8 had interfered with development of the infection by K20 65 strain J Puke strain as indicated previously is slightly more virulent than K20 65 strain in sheep The question arose whether the presumed phenomenon of interference generally occurred between infections by various strains and especially whether an infection by a less virulent strain would inhibit the development of infection by a more virulent strain

A test was carried out in six cattle over one year old to determine whether infec tion indu ed by J–Puke strain could prolong the in ul ation period for K20–65 strain These strains were selected b virtue of the ca of their recognition by the difference in everity of reactions in bovine Four cattle were in culated with J–PuKe strain and three days later two of these four an l tw additional attle with K20–65 strain The result was that the t vo c ttle inoculated with J–Puke strain alone reacted in 7 and 10 days re pectively the two inoculated with K20-65 strain only both in 6 days and the two d uble infected attle caught tick borne fe er da aft r the second inoculati n The react ons in the t o double infected anim l v as ere as tho in the contr ls inoculat l with K20–65 strain It seemed app rent th t although it ha l been allo ed a three day ad antage J–Puke t n uld n t nterf re with the d velopment of K20–65 tr n It is an open que t n wheth pr l ngati n f in cubati n time f r th K 0–65 strain would ha e r ulted f in ul ti n had been delayed unt l the app ran of the reaction b J–Puke t a n

To date it has n t been possible to carry out any more systematic efforts to shed more light on the conditions of the presumed phe nomenon of interference On two occasions inoculation by a hetero logous strain of animals reacting at the time to some other strain has been effected Heifer 13 which had previously reacted to J34 64 strain and subsequently to K1 65 strain was inoculated with J1 65 strain when experiencing relapse to K1 65 strain No reaction to J1 65 strain ensued

However it cannot be said whether this inhibition was attributable to infection by the K1 65 strain or to developed immunity It is suspected that the phenomenon of interference is at least partly responsible for this In the next paper of the series data will be presented to demonstrate that immunity to neither J31 64 nor K1 65 strain alone inhibited development of the reaction by J1 65 strain

Although no control sheep was inoculated simultaneously it was suspected that a slight prolongation of incubation time had taken place when sheep 17 was inoculated with J Puke strain at the end of clinical reaction to K20 65 strain The incubation time for J Puke was 5 days and the following reaction was milder than the preceding one attributable to K20 65 strain

DISCUSSION

No previous mutual comparisons have been made of strains of bovine tick borne fever agent in regard to virulence or other properties It was indicated in the introduction that evidence of the occurrence of differences in virulence in sheep of ovine strains has been presented A strong tendency towards variation appears to be a property common to agents of both ovine and bovine tick borne fever That both the range and the frequency of variation of the tick borne fever agent may be very great was demonstrated on measurement of the virulence in sheep of strains treated in the present study A range from apparent non virulence to high virulence has been presented

Several studies although mostly limited have been made of the virulence of bovine tick borne fever agent in sheep or ovine agent in cattle Neither *Hudson* (6) nor *Foggie & Allison* (3) could regularly infect sheep with the strain of the bovine agent they were investigating *Overas & Hunshamar* (10) induced mild reactions in two sheep with their agent A similar low infectivity of ovine strains for cattle has been recorded (1) However *Hudson* (6) did not mention any difficulties in infecting cattle with a Scottish ovine strain although he did not describe these reactions in detail Thus a higher virulence for the natural host of the strains studied has been a general observation this has led to the postulation of considerable differences between strains associated with bovine or ovine disease under natural conditions (2 3) It has further been considered probable that in a limited area there could only be present either the ovine or the bovine strain of the tick borne fever agent dependent upon whether sheep or cattle constitute the major grazing population (3 19)

Higher virulence in the natural host cattle was also the property of most Finnish strains isolated from bovine field cases Nonetheless two strains (K20 65 and K9 66) appeared to possess about the same degree of virulence in sheep and in cattle The occurrence of this type of strain has not been described earlier It is interesting that no sheep had been

kept on the farms of origin of these strains This finding is thought to indicate that the difference between the variants of the tick borne fever agent which induce natural disease in sheep and those affecting cattle need not in general be so strict as has been thought earlier the occurrence of intermediary forms may be relatively frequent Absence of sheep on the farms on which K20 65 and K9 66 strains originated suggests that development of a strain of high virulence in sheep would not necessarily presuppose an adaptation to sheep but could perhaps also be the results of random variation

Throughout Finland cattle far outnumber sheep (15) According to the theory that either an ovine or a bovine variant of the agents achieves dominance in the area and excludes the other (3 19) a build up of sheep strains would be improbable in Finland Isolation from the ticks of J Puke strain which was highly virulent in sheep and of only low virulence in cattle was therefore surprising Even more surprising was the fact that on the same pasture there was obviously present another strain virulent in cattle (see Material and Methods) From the present experimental results it has emerged that an animal may within a short time be infected with several heterologous strains in the following paper (17) evidence will be given that two heterologous strains may occur even simultaneously in one animal Thus there appears to be no theoretical objection to the possible existence of two clearly different strains on a pasture

It is considered highly improbable that the conspicuous prolongation of the incubation time for K20 65 strain in sheep 8 could have been effected by specific cross immunity induced by heterologous strains no indication had appeared of the operation of such a mechanism in comparable tests with several sheep (The immunological difference of the strains concerned will be well documented by the results presented in the next paper of the series (17)) If it is not specific immunity what then is the nature of the enhanced resistance? Although to date the subject has not been studied to an extent which justifies drawing any conclusions speculation in this connection already appears worthwhile It could be possible that this phenomenon is effected by an interference mechanism not too remote from that described for viruses There are reports that infections by organisms bearing similarities to tick borne fever agent (18) by some rickettsiae induce the formation of interferon like substances (5 8) it has also been reported that infection by *Brucella abortus* produces interferon (20) The point at issue is whether interferon like substances or some other substances like endogenous pyrogen (tick borne fever is characterized by high fever and destruction of granulocytes) could in some way mediate in the inhibition of intracellular multiplication of these bacteria like organisms? The first indication that interferon might in fact do so has been provided by *Hanna Merigan & Jawetz* (4) who demonstrated that virus induced interferon inhibited the multiplication of a TRIC agent

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SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

7 *Technical Aspects*

By

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Received 6 ii 67

Several new type agglutinogens have been described in *Staphylococcus aureus* (3 4 5 6 7 8) The number of type strains has also been extended being now 18 All type strains have been examined by slide agglutination in absorbed rabbit immune sera the so called factor sera (13) Portions of the factor sera have been further absorbed with one strain each and thereafter tested again for residual agglutinins Similar absorptions were carried out with the whole immune sera and new factor sera thus produced were in turn absorbed with each type strain as above until completely exhausted Thereby all 18 type strains were characterized by their agglutinogen pattern their absorbing capacity and their ability to elicit agglutinins in rabbits

In the course of this work it was repeatedly felt that parts of the typing technique required a critical re examination the result of which has been presented here Included is also a summing up of technical modifications reported in earlier articles (5 6)

MATERIALS AND METHODS

Strains and cultivations The 18 serological type strains (3 5 8 13) were grown on nutrient agar slants and mannitol salt agar (Difco) plates overnight at 37 C For demonstration of the h and 760 l antigens mannitol salt agar plates were incubated overnight at 25 C

Immunization agglutination and absorption procedures were the same as before (15) when not described separately under Experiments

EXPERIMENTS AND RESULTS

Among technical problems met with in serological typing of *Staph aureus* four are of major importance False clumping of the bacteria normal agglutinins against staphylococci in rabbits varying antibody response and blocking of antigens

False Clumping

The term false clumping is used here to designate an aggregation of bacterial cells not caused by antibody as opposed to true agglutination

Most colonies of *Staph aureus* are of the smooth type In suspension however the organisms frequently show a tendency to aggregate a troublesome property which in some studies has rendered many strains non typable Moreover for grounds to be presented below there is reason to believe that false clumping has sometimes been misinterpreted as true agglutination

Slide agglutination of staphylococci is performed with colonies taken up with a loop from the agar surface Bacteria deriving from different areas of the agar occasionally show different degrees of stability in suspension

This may require the inclusion of a control suspension with bacteria taken from the same area in every testing of the strain Alternatively a suspension of the organisms can be prepared beforehand for use in all tests The latter procedure will cause further dilution of factor sera and should as a rule be avoided This problem may however be considered of minor importance provided its possible occurrence is borne in mind

Every microbiologist will surely be familiar with the clumping of *Staph aureus* in the presence of fibrinogen and it is hardly necessary to warn against the use of plasma instead of serum in serological typing of this bacterium However this brings up the point of the stability of staphylococci in suspension being greatly influenced by the composition of the suspension fluid A factor serum is an immune serum which has been absorbed with organisms harvested from nutrient agar In addition to specific antibodies and normal serum components the factor serum contains substances from the culture medium as well as materials extracted from the bacteria It is therefore obvious that saline is by no means a satisfactory suspension fluid for the control of false clumping This is illustrated by an example in Table 1

TABLE 1
False Clumping Revealed by the Use of an Absorbed Serum as a Control

Strain	Agglutination in		
	n serum	saline	*n* serum abs w str F21
F21	++	—	++

Code + to +++ Strength of agglutination — No agglutination

It is seen from Table 1 that strain F21 was agglutinated by the *n* serum the saline control being negative However the nature of the clumping gave rise to suspicion of false clumping The *n* serum was therefore absorbed with strain F21 The absorption had no influence on the clumping which accordingly was false although a stable suspension was obtained with saline

Consequently we recommend suspension of the bacteria as a con

trol in a rabbit serum diluted 1 to 10 of which the agglutinins have been exhausted by absorption

As mentioned above it was the character of the clumping that gave rise to suspicion of false clumping. It may be of practical importance to be familiar with some of the features that distinguish false clumping from true agglutination. In false clumping the organisms aggregate in the centre of the drop and have a tendency to sink below its surface. In true agglutination on the other hand the organisms become oriented peripherically in the drop and tend to float. Moreover, false clumping reaches a maximum very soon whereas true agglutination shows a gradual increase in strength on continued rocking of the slide. When both occur concurrently the above mentioned features may provide evidence for the presence of a true agglutination masked by a false clumping. In a strain like F21 which potentially is spontaneously agglutinable a weak agglutination is often augmented by a superimposed false clumping triggered by the agglutination which may then be misinterpreted as a strong reaction.

Several means have been proposed to avoid the false clumping: the addition of antiformin, the autoclaving of the organisms, an increased salt concentration (mannitol salt agar) and the use of fresh young nutrient agar cultures. The two first procedures will alter the milieu in which the agglutination takes place and the sensitivity of the test will then have to be checked for each factor serum. Autoclaving destroys some agglutinogens and cultivation on mannitol salt agar may influence the synthesis of antigens. In this laboratory we have mostly resorted to the use of fresh 5 hour old nutrient agar cultures which are more stable in suspensions than older cultures. As shown by *Oeding* (15) these young cultures may give stronger agglutination but with most factor sera they give the same results as fresh 24 hours cultures. If a 5 hour culture shows false clumping it is transferred to a new culture and tested again. After one or more transfers a stable suspension is usually obtained.

Agglutinins against Staph. aureus *in Pre-Immune Rabbit Sera*

Most of the pre-immune sera of our albino rabbits agglutinate *Staph. aureus* weakly. In the standard procedure (15) the serum is tested in dilution 1:10 for agglutinins against the strain to be used for immunization. If there is little or no agglutination the immunization is started. This gives rise to two questions. Is it sufficient to test the serum with the immunizing strain only? If weak agglutinins are demonstrated can we be sure that the agglutinins are removed by the later absorption for preparation of factor sera and will thus not interfere with the typing?

To examine this question 40 sera from non-immunized rabbits were tested in dilution 1:10 for agglutinins against the 18 *Staph. aureus* type strains. All of the sera agglutinated several strains. Some strains

Wood 46 Cowan II 670 5687 and 6376 were agglutinated by a few sera only or not at all Strain Cowan I on the other hand was agglutinated by all sera The agglutinin patterns indicated the presence of several different agglutinins No attempts were made to differentiate or identify the individual agglutinins

With regard to the first question it is clear from these investigations that pre immune sera must be tested with all type strains

Still more important is the next question Will the normal agglutinins be removed by the absorptions made later for preparation of the factor sera? This was examined by testing the type strains for their capacity to absorb normal agglutinins Five sera representing the different agglutinin patterns were pooled and diluted 1 2 Portions of the pool were absorbed with one type strain each and tested for residual agglutinins The absorbing capacity appeared to vary considerably from one strain to another While the standard absorption dose of strain Cowan I organisms absorbed all agglutinins three times this dose of strain Wood 46 showed almost no absorption When the standard dose of organisms was used eight strains namely strains 28 Wood 46 17A Cowan II Cowan III 1015 830 and 5687 showed inadequate absorption of normal agglutinins

Since little is known about the nature and distribution of normal agglutinins in different colonies and strains of rabbits no general rule can be given as to which strains should be included in the absorptions for preparation of factor sera Before inoculation the serum should therefore be tested in dilution 1 10 with all type strains Thereafter the absorbing capacity for possible agglutinins is tested with the strain or strains to be used later for absorbing the immune serum

TABLE 2

Agglutination of Type Strains in a Pre Immune Serum before and after Absorption

Strain	Agglutination in pre immune serum (1 10) not abs	abs with st [illegible]1
1503	+	—
28	++	—
17A	++	—
Cowan I	+++	
830	+	—
Other type strains	—	—

Code see Table 1

An example illustrates this (Table 2) It appears from Table 2 that six strains were agglutinated by the pre immune rabbit serum diluted 1 10 The resulting immune serum is intended for preparation of factor *a* serum which is made from antiserum to strain 3647 by absorbing

with strain F21 The absorbing capacity of strain F21 for normal agglutinins is therefore tested It is seen from Table 2 that all agglutinins were removed by this absorption We can conclude that this rabbit can be used for immunization with strain 3647 for preparation of factor *a* serum

It was commonly observed in these experiments that several strains showed false clumping when normal sera were used in a concentration greater than 1 10 In some sera strain Cowan I also showed false clumping in dilution 1 10 It is likely that false clumping such as this is due to special physico chemical properties of the serum In serological typing of staphylococci we must therefore advise against using factor sera more concentrated than 1 10 The latter observation also adds further support to our opinion expressed above that a saline suspension is not a satisfactory control

TABLE 3

Antibody Composition of Two Rabbit Sera (K727 and K728) after Immunization with Strain 3647

Antibodies against	Serum K727	Serum K728
a_1	++	—
a_2	++	—
a_3	++	+
a_4	+++	+++
a_5	++	++
b_1	+	+++
c_1	(+)	+++

Code (+) to +++ denote content of agglutinins — Agglutinins not demonstrated

Immunization Problems

Rabbits vary considerably in antibody response to staphylococcal antigens Some rabbits are generally poor antibody producers others produce one antibody abundantly but little of another The latter is illustrated in Table 3 Two rabbits were immunized with strain 3647 a strain frequently employed for immunization The rabbits were given parallel injections from the same batch of organisms It is seen from the table that both rabbits produced potent agglutinins against antigens a_4 and a_5 the antibody composition varying widely in other respects Agglutinins to a_1 and a_2 could not be demonstrated in serum K728 which on the other hand had strong b_1 and c_1 agglutinins In order to save time several rabbits should therefore be immunized simultaneously The complete antibody composition of an immune serum will sometimes be of interest because a serum which cannot be used for the *planned factor serum may appear to be suitable for preparation of* another

The results of an immunization depend primarily on the selection

of the right strain The presence of a strong agglutinogen in a strain is no guarantee of a successful immunization A strain which is not agglutinated at all may appear to be the best one for production of the respective antibody and it appears that the choice of strain is based on practical experience

If there is a poor antibody response after one series of injections further immunization will often be of no use After a successful immunization maximal amounts of serum should be withdrawn since a later booster injection may produce different agglutinin patterns

Blocked Antigens

It is the author s opinion that the greatest difficulty in serological classification of staphylococci is the common occurrence of blocked agglutinogens Antigen blocking means that under certain conditions antigenic groupings are not free to react with the agglutinin The cause of this is not known most likely it is steric hindrance The same type of blocking is met with in strains of *E coli* and salmonella (blocking of somatic antigens by K and Vi antigens)

TABLE 4
Methods Used for Unmasking of Blocked Antigens in Staphylococci

1 Treatment with trypsin (14)
2 Autoclaving (1 14 16)
3 Fresh 5 hour old nutrient agar cultures (15)
4 Mannitol salt agar cultures 37 C (6)
5 Mannitol salt agar cultures 25 C (6)

Many methods have been tried for unmasking of blocked antigens A list of methods used in typing of staphylococci has been presented in Table 4 Treatment with trypsin (14) is time-consuming destroys protein antigens and may cause false clumping *Oeding* (14) used both autoclaved and live bacteria in his early work since autoclaving revealed antigens which could not be demonstrated by means of live organisms Later (15) he found that autoclaving could be omitted when he used fresh young cultures preferably 5 hours old When several new staphylococcal antigens were discovered (6) it soon became clear to the author that 5 hour cultures could by no means replace autoclaved bacteria Autoclaving was not however a very sensitive method for the revelation of blocked antigens In a search for newer methods mannitol salt agar cultures of the bacteria grown at 37 and 25 C (6) were found to give stronger reactions than autoclaved organisms Moreover the method was much simpler

Agglutinogen blocking as met with in staphylococci is illustrated in Tables 5 and 6 It is seen in Table 5 that a live nutrient agar culture of strain 3647 was not agglutinated by *a* serum in contrast to autoclaved

with strain F21. The absorbing capacity of strain F21 for normal agglutinins is therefore tested. It is seen from Table 2 that all agglutinins were removed by this absorption. We can conclude that this rabbit can be used for immunization with strain 3647 for preparation of factor *a* serum.

It was commonly observed in these experiments that several strains showed false clumping when normal sera were used in a concentration greater than 1:10. In some sera strain Cowan I also showed false clumping in dilution 1:10. It is likely that false clumping such as this is due to special physico-chemical properties of the serum. In serological typing of staphylococci we must therefore advise against using factor sera more concentrated than 1:10. The latter observation also adds further support to our opinion expressed above that a saline suspension is not a satisfactory control.

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development of a set of type strains Our 18 type strains have been mapped antigenically by agglutinogen patterns absorbing capacity and agglutinin production in rabbits We have introduced an additional culture medium mannitol salt agar and in some instances the bacteria are grown both at 37 and 25 C In this way the method has been rendered considerably more sensitive by means of a simple and safe modification As might be expected the absorption experiments revealed some weak agglutinogens which could not be demonstrated by agglutination

The main problem is undoubtedly the commonly occurring blocking of antigens The *h* antigen for instance cannot be detected by the introduction of a tube test by a prolonged incubation or by a finer reading since it is blocked in most strains grown on nutrient agar

Cultivation on mannitol salt agar was found to be superior to autoclaving in unmasking of the blocking The mannitol salt medium differs from nutrient agar in the high sodium chloride concentration (8 5 per cent) and the mannitol content of the former Mannitol is oxidized by *Staph aureus* resulting in acid formation As reported in an earlier article (6) the presence of mannitol and the low pH have no influence on the typing and the unmasking effect was found to be due to the high salt concentration We have however preferred to employ mannitol salt agar since this medium is commonly used in laboratories

The nature of the blocking of antigens in staphylococci has not been established On the basis of the results of trypsinization and autoclaving of the bacteria *Oeding* (14) proposed a model for the antigenic build up of a staphylococcus an outer protein and an inner polysaccharide layer How blocked antigens are revealed by cultivation at a high salt concentration and/or at low temperature is not clear If we presume an outer protein layer it is likely that the salt and the low temperature inhibit the synthesis of these proteins thus exposing the polysaccharide layer

On the basis of our present experience with the type strains the following procedure is proposed for demonstration of agglutinogens Bacteria grown on mannitol salt agar for 18 hours at 25 C are used for testing in factor sera *a* c_1 *h* l_1 and 670–1 For use with the remaining sera the bacteria should be cultivated on nutrient agar for 18 hours at 37 C The results of typing of many other strains must be awaited before more definite directions can be given

The staphylococcal agglutinogens known at present have been identified by examining absorbed immune sera for agglutinins against the 18 type strains The number of immune sera examined as well as the number of strains employed for absorption are limited although the strains are selected from many others tested If other strains and more rabbits are used for immunization new agglutinogens will most probably be found Neither can the possibility be excluded that our present sera contain other agglutinins which might be detected if other strains

were introduced for absorption. However, if further progress is to be made in the serological classification of staphylococci, it is at present more important to standardize the technique and to get more experience and information about the value of the new factor sera in practical typing work.

We are inclined to emphasize that those who adopt our method should be familiar with the agglutination patterns of each type strain and check all factor sera as indicated under Summary. Any alteration may indicate interference by other agglutinating factors and should be cleared up to avoid conflicting results. An exchange of strains between laboratories for comparison of typing results is to be recommended.

SUMMARY AND CONCLUSIONS

The technique for serological typing of *Staph. aureus* has been critically examined. The following general technical procedures are recommended as a supplement to *Oeding's* (15) standard method.

1. *Immunization*. a) Pre-immune sera diluted 1:10 should be tested with all type strains.

b) The strains to be employed in later absorption of the immune serum should be capable of removing all agglutinins in the pre-immune serum.

c) Booster injections should be avoided.

2. *Agglutination*. a) As a control for false clumping, saline is replaced by an exhausted immune or pre-immune rabbit serum diluted 1:10.

b) Agglutination is carried out with bacteria grown for 18 hours on nutrient agar at 37° C and on mannitol salt agar at 25° C.

Further details are given above.

c) Before use a factor serum should be checked with all type strains.

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AN IN VITRO STUDY OF THE ANTIBIOTIC SENSITIVITY OF *FLAVOBACTERIUM MENINGOSEPTICUM*

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Flavobacterium meningosepticum is a gram negative rod first described by *Elizabeth O King* in 1959 Since then six serologically different types termed A–F have been found The microorganism was isolated from blood and spinal fluid of premature infants with severe meningitis In total reports of 63 such cases have been published (*Brody et al* 1958 *Vandepitte et al* 1958 *Cabrera & Davis* 1961 *George et al* 1961 *Seligmann et al* 1963 *Sugathadasa & Arseculeratne* 1963, *Vandepitte et al* 1965 *Watson et al* 1966) In addition the microorganism has been isolated postoperatively from the blood of adult individuals Prognoses in these cases were fair (*Olsen et al* 1965)

In the individual studies the antibiotic sensitivity has been found to vary *Vandepitte et al* (1965) found sensitivity to streptomycin and tetracycline and resistance to all other antibiotics All other investigators have found sensitivity to erythromycin and chloramphenicol and resistance to penicillin and polymyxin In addition *King* (1959) found resistance to tetracycline streptomycin neomycin and bacitracin while others have found sensitivity to tetracycline and streptomycin (*Vandepitte et al* 1958 *Sugathadasa & Arseculeratne* 1963) *Watson et al* (1966) found sensitivity to neomycin and resistance to streptomycin tetracycline and ampicillin No data concerning the method used by the last mentioned authors The other investigators mentioned used an agar diffusion method

Although sensitivity to one or several antibiotics has been found for all cases therapeutic results have been poor Among 51 well documented cases the outcome was fatal in 38 and absence of sequelae was seen only in four patients

The object of this study has been on the basis of *in vitro* experiments to form an idea of the antibiotic treatment which could be expected to be most effective against infections caused by *Flavobacterium meningosepticum*

The author thanks Miss *Lone Nielsen* for valuable technical assistancee

METHODS

1 *Estimation of 50 per cent Inhibitory Concentration (IC 50)*

Two two fold dilution series of an antibiotic dissolved in broth were produced Each tube contained 0 5 ml and the first tubes generally holding 1000 µg/ml of antibiotic The tubes were inoculated by means of a platinum loop and incubated for two days at 35 C upon which blood plates were inoculated using matter from all tubes without visible growth in order to determine whether the effect was of a bactericidal or bacteriostatic nature

From the number of tubes with growth a titre was estimated according to Karbers method and the IC 50 was calculated by the equation

$$\text{IC } 50 = \frac{1000}{2^{10-T}} \sim 2^{T}$$

where T is the estimated Karber titre when the initial tube is termed 10 and contains 1000 µg/ml antibiotic

The inoculum estimated in all tests was 10^4–10^5 per tube The size of the inoculum was estimated by production of a ten folddilution series of the bacterial culture followed by inoculation of 0 1 ml onto blood agar plates

Among a total of 314 double series 256 gave the same end point for the two series in 55 series the difference was one tube and in three there was a difference of two tubes If a double series is considered as a double estimation of the end point the standard deviation of the estimation can be calculated to 0 23 ($f = 314$)

2 *Combination of Two Antibiotics*

a *Combination of two antibiotics with IC 50 > 40 µg/ml* If the antibiotics to be studied in pairs were termed A B and C the tubes should be placed according to the following scheme (*Garrod & Waterworth* 1962)

AA	AB	AC
	BB	BC
		CC

As continuous serum concentrations above 20 µg/ml of an antibiotic cannot be expected a tube of type AB was made to contain 20 µg/ml of A + 20 µg/ml of B Tubes of type AA contained 40 µg/ml of A The tubes were inoculated and incubated for two days at 35 C In tubes without growth a potentiating effect of the two antibiotics must be present Such combinations were further studied using the method described later under c

b *Combination of antibiotics with IC 50 > 40 µg/ml and an antibiotic with IC 50 < 40 µg/ml* A number of two fold dilution series were produced using an antibiotic A with an IC 50 < 40 µg/ml One double series contained antibiotic A exclusively but 20 µg/ml of all antibiotics with an IC 50 > 40 µg/ml were added to the remaining double series Subsequently they were inoculated and incubated at 35 C for two days If upon addition of an antibiotic B a fall in the titre was found synergism between A and B must be present An increase in the titre indicates antagonism between A and B

A fall of one log unit in the titre indicates that 20 µg/ml of antibiotic B added replaces up to 50 per cent of IC 50 of antibiotic A A fall in the titre exceeding one log unit indicates the presence of a potentiating effect

All antibiotic combinations showing a fall in the titre after incubation for two days were further studied using the method described under c

c *Combination of two antibiotics with IC 50 < 40 µg/ml* Of the two antibiotics A and B that were to be combined a two fold dilution series was produced Next 64 tubes were placed in a check pattern Tubes recorded in column furthest to the right contained antibiotic A exclusively in a two fold dilution series and those recorded in the lowermost horizontal row contained antibiotic B exclusively in a two fold dilution series The remaining tubes contained A and B in all concentrations that could be produced by combinations of the two dilution series The tubes were inoculated and incubated for two days at 35 C upon which the number of tubes with visible growth was recorded

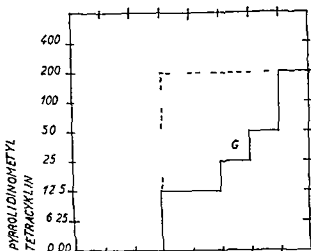


Fig 1

A diagram depicting results obtained by a combination of erythromycin/pyrrolidino methyl tetracy line using method ? c The figures represent concentrations in μg/ml of the two antibiotics Growth appeared in the tubes below and to the right of the polygonal line Tube G contains 1/8 of the least inhibitory concentration of both antibiotics thus a potentiating effect is present

Using suitable initial concentrations zones without growth will be present to the left and in the upper part of the system as concentrations at these sites should exceed the lowest inhibitory concentrations of the two antibiotics In the following figures these zones are seen to the left and above the dotted line An appearance of growth in these zones indicates an antagonism between the two antibiotics (Fig ?)

If a tube without growth is found below and to the right of the two zones synergism is present (Fig 1) If the concentrations of the two antibiotics are ex pressed in fractions of the lowest inhibitory concentration a measurement of the synergist c effect will be obtained in the sum of these fractions in the tube without growth in which this sum is smallest.

A definite potentiating effect will be present if a tube without g owth is found in which both antibiotics have been diluted at l ast two quares (four fold) from their *lowest inhibitory concent ations*

In order to obtain an expression of the combination effect e luated on the basi of a bactericidal point of iew all tubes without visible growth were used for in oculation onto blood ga plates

Strains Employed

l ing s six se ologi ally different strains were u ed They h e all be n is lated from premature infants with m ningitis

A King no	14	ATCC 13?53
B l ing s no	4??	ATCC 13 4
C King s no	3375	ATCC 13 55
D K ng s no	f9?5	
E King s no	8?88	
F l ng s no	8707	

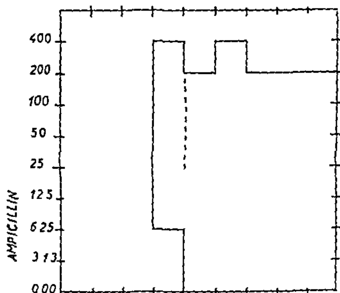


Fig 2

A diagram depicting results obtained by a combination of vancomycin/ampicillin Antagonism exists as growth appeared in several tubes in zones where the *lowest* inhibitory concentration of the one antibiotic was exceeded

In addition the following strains were studied which were all isolated from the Department of Thoracic Surgery Odense City and County Hospital

5233 isolated from blood of a patient (j no 267/64)
6692 isolated from blood of a patient (j no 456/64)
6896 isolated from blood of a patient (j no 390/64)
T 396 isolated from a bronchoscopic suction apparatus
L 5 isolated from the air in an operating theatre

The biochemical properties of the strains were described by Kinj (1959) *Olsen et al* (1965) and *Olsen* (1966)

RESULTS

The 50 per cent inhibitory concentrations (IC 50) found are recorded in Table 1 The log IC 50 represents mean values N indicating the number of tests from which the mean values were calculated The IC 50 were calculated on the basis of the *mean* values and recorded in µg/ml

The following antibiotics were found to be bactericidal Vancomycin penicillin ampicillin and streptomycin The remaining ones were found to have a bacteriostatic effect In the novobiocin tests numerous surviving colonies were found even at high concentrations whilst the other bacteriostatic antibiotics produced a bactericidal effect in concentrations two to four times that of the inhibitory concentration

TABLE 1a

Determination of Sensitivity of 11 Flavobacterium meningosepticum *Strains*

	Erythromycin lactobionate			Novobiocin sodium (Albamycin)			Vancomycin hydrochloride			Fucidin			Benzyl penicillin sodium		
	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N
King A	11	3 5	1	5 5	2 5	3	6 8	2 8	2	15	3 9	4	34	5 1	3
King B	6 3	2 7	5	7 8	3 0	4	31	5 0	2	17	4 1	4	38	5 3	3
King C	5 9	2 6	7	9 0	3 2	3	11	3 5	3	54	5 8	6	24	4 6	7
King D	3 2	1 7	4	36	5 2	3	19	4 3	2	31	5 0	3	58	5 9	3
King E	3 0	1 6	6	3 9	2 0	4	6 8	2 9	4	11	3 5	4	29	4 9	6
King I	5 2	2 4	5	7 8	3 0	2	14	3 8	3	5 0	2 2	3	95	6 6	3
Sm		0 87			0 84			0 88			0 72			0 54	
I 5	16	4 0	2	5 5	2 5	2	16	4 0	2	11	3 5	2	51	5 7	2
5933	1 7	0 8	3	2 0	1 0	2	9 8	3 3	2	22	4 5	2	58	5 9	3
6192	2 0	1 0	2	2 8	1 5	2	22	4 5	2	31	5 0	2	72	6 2	2
6886	2 0	1 0	2	3 9	2 0	2	38	5 3	2	31	5 0	2	51	5 7	2
I 396	1 7	0 8	2	3 9	2 0	2	9 8	3 3	2	22	4 5	2	51	5 7	2
Sb		0 60			0 71			0 92			0 71			0 41	

TABLE 1 b

Determination of Sensitivity of 11 Flavobacterium meningosepticum Strains

	Pyrrolidino methyltetracycline (Reverin®)			Ampicillin			Chloramphenicol			Streptomycin			Sulphadimidine		
	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N	IC 50 µg/ml	log IC 50	N
King A	107	8 2	3	77	6 3	2	54	5 8	3	268	8 1	3	6061	12 0	3
King B	51	7 1	3	95	6 6	2	51	5 7	3	379	8 6	1	5654	12 5	3
King C	54	7 2	3	67	6 1	2	98	6 5	3	218	7 8	3	4286	12 1	3
King D	107	8 2	4	154	7 3	2	45	5 5	2	190	7 6	3	4571	12 2	5
King E	82	7 8	6	47	5 6	2	39	5 3	3	190	7 6	6	3021	11 6	5
King F	77	7 7	3	134	7 1	2	14	3 8	3	379	8 6	4	4986	12 1	3
sm		0 93			0 65			0 58			0 50			1 09	
L 5	44	6 9	2	154	7 3	2	88	6 5	2	287	8 2	2	1516	10 6	2
5933	61	7 4	2	95	6 6	2	77	6 3	2	287	8 2	2	2457	11 3	2
6692	61	7 4	2	109	6 8	2	63	6 0	2	144	7 2	2	1741	10 8	2
6386	61	7 4	2	95	6 6	2	88	6 5	2	102	6 7	2	2143	11 1	2
T 396	61	7 4	2	154	7 3	2	63	6 0	2	574	9 2	2	1741	10 8	2
st		0 69			0 50			1 01			2 18			0 94	

Reverin® contains 37 5 per cent pyrrolidinomethyl tetracycline The values recorded have been corrected for this

In order to evaluate the error involved in the method used the following statistical analysis was carried out

The strains 5933 6699 6886 and T 396 were considered to be identical as they had been isolated during one and the same hospital epidemic and were biochemically identical. L 5 was found to be more resistant to erythromycin and therefore excluded from the following calculations

In the four identical strains the standard deviation s_b of log IC 50 was calculated for each antibiotic These values are recorded nethermost in Table I The variances were tested according to Bartlett s test (se for instance *Hald* 1948) and did not differ significantly providing that the streptomycin value was excluded (χ = 4 90 f = 8 P > 0 50) The greater variance noted in the case of streptomycin may be due to an experimental error A mean standard deviation s_{bf} = 0 74 applying to the above mentioned four identical strains was thus obtainable if the streptomycin variance was excluded

Correspondingly as regards the six meningitis strains a mean standard deviation s_{mf} = 0 87 was estimated First the standard deviation of log IC 50 was calculated for each antibiotic as above These values are recorded in the middle line in Table I Bartlett s test showed that values obtained for the six different strains as well as values for the different antibiotics did not differ significantly (χ = 11 72 f = 8 P > 0 05)

Only the streptomycin variance differed significantly in the test

$$F = \frac{s_m^2}{s_b^2}$$

(F = 19 0 f_1 = 4 f = 16 P < 0 0005) and for the remaining antibiotics a mean standard deviation s_{bm} = 0 81 was calculated

Combination of Two Antibiotics

The following tests were carried out on King s serological type C strain the one most frequently isolated from premature children with meningitis

Tests according to method 2a Fucidin pyrrolidinomethyl tetracycline ampicillin chloramphenicol streptomycin and sulphadimidine were combined in all possible combinations using 20 µg/ml of each antibiotic as described under method 2a The culture became sterile only after combination of fucidin/chloramphenicol This combination was further studied using the method described under 2c

Tests according to method 2b Table 2 shows the results after one and two days obtained by this method As an expression of antagonism an increase in the titre of one log unit was found after two days using the combinations vancomycin/ampicillin and novobiocin/sulphadimidine In streptomycin/erythromycin a titre increase of ½ log unit was found A fall in the titre was seen in a number of combinations most marked in the case of erythromycin/fucidin Combinations involving a fall in the titres after two days were also studied in accordance with the principles of method 2c

Tests according to method 2c Figs 1 and 2 illustrate results of the combinations erythromycin/pyrrolidinomethyl tetracycline and vancomycin/ampicillin Fig 1 depicts a potentiating effect Fig 2 an antagonizing effect

TABLE 2

Results Obtained by Method 2b Combination of Two Antibiotics against Flavobacterium meningosepticum (King's Serological Type [illegible])

	day	log IC 50	log IC 50 after addition of 20 μg/ml of the following antibiotics					
			Fucidin	Chlor amphenicol	Ampicillin	Pyrrolidino methyl tetracycline	Streptomycin	Sulpha dimidine
Erythromycin	1	2 5	0 5	1 5	2 5	1 5	2 5	2 5
	2	3 5	1 5	3 5	3 5	2 5	4 0	3 5
Novobiocin	1	3 5	0 5	2 5	2 5	1 5	3 0	3 0
	2	3 5	3 5	3 5	3 5	3 5	3 5	4 5
Penicillin	1	4 5	2 5	4 5	3 5	4 5	3 5	4 5
	2	4 5	3 5	4 5	4 5	4 5	4 5	4 5
Vancomycin	1	5 5	3 5	4 5	5 5	5 5	5 5	5 5
	2	5 5	4 5	5 5	6 5	5 5	5 5	5 5

Values in the column to the left represent log IC 50 of the four antibiotics stated in the column. Values recorded along the horizontal line represent log IC 50 after addition of 20 μg/ml of the six antibiotics recorded along the uppermost line

TABLE 3

Results Obtained by a Combination of Two Antibiotics Using Method 2c

	Vancomycin	Fucidin	Penicillin	Chlor amphenicol	Novobiocin	Pyrrolidino methyl tetracycline
Erythromycin	S (1/2+1/4)	S (1/8+1/4)	0	S (1/2+1/64)	S (1/2+1/8)	S (1/8+1/8)
Penicillin	A (1+1/16)	S (1/2+1/8)		0	S (1/4+1/4)	0
Chloramphenicol	S (1/2+1/4)	S (1/8+1/4)				
Novobiocin	S (1/8+1/2)					
Ampicillin	A (1/64+1)					
Fucidin	S (1/4+1/4)					
Streptomycin			S (1/4+1/4)			

S synergism A antagonism O indifference Fractions in brackets denote the fraction of the lowest inhibitory concentration of the two antibiotic found in the tube without growth in which the sum of the fractions is smallest

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IDENTIFICATION OF *NEISSERIA GONORRHOEAE* BY MEANS OF FLUORESCENT ANTIBODY TECHNIQUE

By

INGA LIND

Received 4 ii 67

The use of Coons immunofluorescence technique (3 4) in the identification of *Neisseria gonorrhoeae* was first reported by *Deacon et al* 1959 (11) Subsequently further investigations in this field have been performed (6 7 12 15 20 31 34) but so far the fluorescent antibody technique has not been commonly used in the diagnosis of gonorrhoea This technique and its possibilities as a routine method have been the subject of the following experiments the preliminary results of which have been published previously (26)

MATERIALS AND METHODS

Preparation of Rabbit Anti Gonococcal Serum

Selection of rabbits Young rabbits (1700–1900 g) with a negative gonococcal complement fixation test (25 36) were selected for immunization Samples of blood were collected and treated in the same manner as the corresponding samples of antisera

Preparation of antigen The antigen for immunization was made either from a single strain or from a pool of 20–30 strains of gonococci Both fresh strains and well known stock strains were used The strains were grown for 16–18 hours at 36 C in a moist atmosphere containing about 10 per cent CO The medium was the fermentation medium described by *A Reyn* (39) except that sugar phenol red and co-carboxylase were omitted In some cases 2 units of Mycostatin® per ml were added to inhibit moulds The bacteria were harvested in phosphate buffered saline (PBS) pH 7 2 (one litre made from 75 ml of 0 15 mol KH_2PO_4 175 ml 0 15 mol Na_2HPO_4 and 750 ml 0 9 per cent NaCl) containing 3 per cent formalin (i e 3 ml solution formaldehyd plus 97 ml PBS) They were left for 30 minutes at room temperature washed three times in PBS pH 7 2 and then resuspended in 0 3 per cent formalin in PBS pH 7 2 to a density corresponding to 2×10^9 bacteria/ml

Immunization Schedules

A Groups of four to eight rabbits were immunized by intravenous injection of a dose increasing from 0 25 ml to 1 00 ml twice a week for four weeks Five to six days after the last injection blood samples were collected by heart puncture After a pause

Strains of *Staphylococcus aureus* were kindly supplied by *Kirsten Rosendal* M D Department of Diagnostic Bacteriology strains of *Escherichia coli* by *Ida & Frits Ørskov* M D International Escherichia Centre and strains of *Streptococcus pyogenes* by *Beate Perch* Ph D Streptococcal Department (all Statens Seruminstitut) For the clinical specimens I wish to thank *A Perdrup* M D Rudolph Berghs Hospital Copenhagen and *Henning Schmidt* M D Rigshospitalet Copenhagen

containing 10 per cent CO Next morning duplicate smears were made in a drop of tap water on carefully cleaned slides and the microscopic fields were marked with a glass diamond Colonies suspected to be gonococci and/or several spots in confluent growth were touched with a platinum needle and transferred to the slides to form an appropriately thin smear When no growth was visible the surface of the medium was scraped gently with the needle to catch any growth not yet visible to the naked eye (the lag phase may be prolonged for organisms which have been transported for some time) The smears were allowed to dry in the air they were fixed for 5 minutes in methanol dried and rehydrated in PBS pH 7 2 for 10 seconds

Performance of the Test

The smears were placed in a moist chamber and the field covered with 1–2 drops of a mixture of conjugate and a pool of unlabelled normal rabbit sera or rabbit anti staphylococcal sera the optimal ratio having been determined in preliminary experiments Final dilutions of 1 8 of the conjugate and of 1 4 of the unlabelled serum were often found suitable This modification known as the one step inhibition test" (16 35) eliminated most of the non specific background staining and also the reaction with certain other microorganisms especially certain staphylococcal strains which may be a source of error The conjugate was diluted in saline when used without mixing with unlabelled serum

The smears were incubated for 30 minutes at room temperature rinsed thoroughly with PBS pH 7 2 using a washing bottle and placed for 10 minutes in PBS pH 7 2 The slides were dried with filter paper outside the field and mounted with phosphate buffered glycerol (9 parts of glycerol plus 1 part of PBS pH 8.2) Direct smears from secretions were trated in the same way except that they were mounted with a larger cover glass to increase the field for microscopical examination

If not read on the same day the preparations could be kept at 4 C for 3–4 days without any significant alteration in the degree of fluorescence taking place

Microscopy

The microscopes were a Zeiss Standard and a Zeiss Universal microscope fitted with an ultra darkfield condenser and oil immersion objectives (apochrom 40 m I" and apochrom 100 m I) The light source was an Osram HBO 200 high pressure mercury lamp the exciter filters were BG 38 and BG 12 (3 mm) and the barrier filter was No 47 or No 50 depending upon whether or not it was desirable to distinguish the blue autofluorescence exerted by several microorganisms When an ultra darkfield condenser is used only slides with a thickness of 1 mm or less can be applied

The degree of fluorescence was recorded in values from 0 to ++++ The values +++ and ++++ corresponded to the brilliant yellow green fluorescence which was observed in a specific staining reaction The specific positive reaction was characterized by the morphology of the microorganisms *in casu* typical diplococci and the degree of fluorescence (+++ and ++++)

RESULTS

A *Evaluation of the Specificity of the Fluorescent Antibody Test*

1 *Variations in the Specific Staining Reaction*

The picture observed in the fluorescence microscope when a pure culture of gonococci was stained with FITC labelled antigonococcal globulin was found to vary in the following aspects

a) *Age of culture* Gradually as a culture grew older the [illegible] picture of brilliantly fluorescent diplococci became blurred the degree of fluorescence diminished and many abnormal forms were seen together with diffuse weakly stained masses After 18 hours growth cul

tures produced suitable preparations after 72 hours lysis was nearly complete When primary cultures of material from patients were examined 24 hours growth was suitable presumably on account of a longer lag phase and in some cases perhaps also on account of some inhibitory effect of substances in the secretion from the patient (*e g* antibiotics)

Deacon et al suggested that subcultures would not be usable in the FAT since the reaction was thought to be due to a very labile surface antigen which was lost on subculturing (11) In order to investigate this suggestion three freshly isolated strains were selected which showed brilliant fluorescence of a thick even layer on each cell in the smear These strains were subcultured daily From each subculture smears were prepared for the FAT After 16 transfers no alterations in the morphological picture nor in the degree or localization of the fluorescence were observed

b) *'Serotypes' of the strains* Antisera against single strains or pools of strains produced after both schedule A and schedule B gave good conjugates in all cases However each individual conjugate showed small variations in reactivity when tested against a large number of strains No conjugate could be diluted further than 1 10 if a + + + or + + + + reaction was required also in relatively thick smears of gonococci Single strains with their homologous conjugates gave a + + + + reaction in higher dilutions (1 40 1 80) These values were read at a magnification of 800 and a FITC protein ratio of 1 40 during the labelling procedure

c) *Batch of FITC* Different products of the dye produced conjugates which exerted varying degrees of fluorescence Variation in the time and conditions of storage of the dye before use might also be of significance (24)

d) *Antibiotic treatment of the patients and use of antibiotics in the medium* It is known that subinhibitory concentrations of antibiotics may induce morphological changes in gonococci (30) Most of such forms were well stained in the FAT When they appear together with diplococci of normal appearance they do not cause diagnostic difficulties On the other hand such forms are not likely to be recognized as gonococci in direct smears Such strains in which morphological changes are brought about by the antibiotics in the medium are very rare

2 *Staining of* Neisseria *(N) other than* N gonorrhoeae

The strains shown in Table 1 were examined with two labelled rabbit antigonococcal sera I) A pool of antisera against a stock strain of *N gonorrhoeae* assumed to possess a comparatively high content of protein antigens (37 42) II) A pool of antisera prepared against a pool of 20 freshly isolated gonococcal strains

TABLE 1
Stainability with FITC Labelled Antiserum to N. gonorrhoeae Species within the genus Neisseria.

Strains		Stained with FITC labelled antiserum to: N. *gonorrhoeae* SS 19377/40 I	Pool of 20 strains of N. *gonorrhoeae* II	II mixed with unlabelled normal rabbit serum
N. *meningitidis* group A	ATCC 13077	N	+ → +++	N
N. *meningitidis* group B	ATCC 13090	N	N	N
N. *meningitidis* group B	ATCC 13091	N	N	N
N. *meningitidis* group C	ATCC 13102	N	N	++ → +++
N. *meningitidis* group D	ATCC 13113	N	+++	+++
N. *meningitidis* 16 strains isolated at SS		+ → ++++	0 → +++	0 → +++
N. *catarrhalis*	ATCC 8193	N	+++	N
N. *catarrhalis*	ATCC 8176	N	+++	N
N. *catarrhalis*	ATCC 7900	N	–	–
N. *catarrhalis*	NCTC 4103	N	–	–
N. *catarrhalis*	SS 465/2	N	N	N
N. *catarrhalis*	SS 87895	N	+++	N
N. *flava*	ATCC 14221	+++	+++	N
N. *perflava*	ATCC 10555	N	N	N
N. *subflava*	ATCC 11076	N	+++	N
N. *flavescens*	ATCC 13120	–	N	N
N. *sicca*	ATCC 9913	–	N	N
N. *haemolysans*	ATCC 10379	N	N	N
N. *cuniculi*	ATCC 14688	N	N	N
N. *cuniculi var gigant*	ATCC 14689	N	N	N
N. *canis*	ATCC 14687	N	N	N
N. *caviae*	ATCC 14659	N	N	N
N. *denitrificans*	ATCC 14686	N	N	N
N. *species*	SS 78438	N	–	–
N. *gonorrhoeae* (URI)	ATCC 11688	–	N	N
N. *gonorrhoeae* (J G)	ATCC 11689	–	N	N
N. *gonorrhoeae* (fresh control)		++++	++++	++++

++++ Brilliant capsule like fluorescence typical appearance of specific staining
+++ Ditto but less brilliant
0 → +++ Variation in degree of fluorescence of bacteria in the same pure culture
N Negative: no or weak fluorescence
– Not examined
SS Statens Seruminstitut, Copenhagen Denmark

Furthermore the strains were tested in a one step inhibition test where conjugate II was mixed with the corresponding unlabelled serum from pre immune animals (normal rabbit serum).

As was to be expected the first species examined N. *meningitidis* showed a high degree of cross reactivity. This was most pronounced with the polyvalent antiserum and was not inhibited by normal rabbit serum.

The second species N. *catarrhalis* which is also serologically related

to *N. gonorrhoeae*, did not cross react with conjugate I but some of the strains did so with the polyvalent conjugate II. This reaction was inhibited more or less by normal rabbit serum but in all cases to such a degree that the picture did not resemble a specific reaction.

In the third series which consisted of serologically less related strains only *N. flava* and *N. subflava* showed some cross reactivity; in both cases the staining was inhibited by the addition of unlabelled normal rabbit serum.

The cross reaction with *N. meningitidis* was the only which was not inhibited by normal rabbit serum. The staining of meningococci was therefore tested both after absorption of the antigonococcal serum prior to labelling and after absorption of the antigonococcal conjugate. Formalin killed suspensions of *N. meningitidis* Group A and a pool of *N. meningitidis* Group A, B, C and D were used for the absorption. By these procedures it was possible to prevent the staining of meningococci but at the same time the staining of gonococci was markedly reduced and the variability in the degree of fluorescence of different gonococcal strains was increased. Absorption with various tissue powders caused a decrease in the staining intensity of both gonococci and meningococci.

TABLE 2

Stainability with FITC Labelled Antiserum to N. gonorrhoeae Species Considered as a Source of Error in Gram Stained Smears

Strains		Stained with FITC labelled antiserum to		
		N. gonorrhoeae SS 19937/40 I	Pool of 20 strains of *N. gonorrhoeae* II	II mixed with unlabelled normal rabbit serum
Mima polymorpha	ATCC 9957	N	N	N
Mima polymorpha	ATCC 10973	N	N	N
Herellea species	ATCC 9955	N	N	N
Herellea species	ATCC 9956	N	N	N
Herellea species	ATCC 11959	N	N	N
Moraxella bovis	ATCC 10900	N	N	N
N. gonorrhoeae (fresh control)		++++	++++	++++

++++ Brilliant capsule like fluorescence, typical appearance of specific staining
N Negative, i.e. no or weak fluorescence

3. *Staining of Species not Belonging to the Genus Neisseria*

Table 2 shows a series of microorganisms which in Gram stained smears often have been considered as sources of error in the diagnosis of gonorrhoea. It will be seen from the table that these bacteria did not cause any difficulty in the FAT.

Table 3 shows the results of examination of microorganisms the majority of which may occur in cultures from specimens to be examined

for gonococci The main problem was caused by certain staphylococcal strains because of the similarity in morphology and the intensity of staining The reaction was eliminated by the admixture of normal rab bit serum Furthermore such strains never occurred in specimens re ceived for identification of gonococci except the one which revealed the problem and which originated from a male urethra One hundred and one strains of *Staphylococcus albus* were tested These were iso lated from cultures intended for the delayed FAT Since no *Staphylo coccus aureus* were found in these cultures all the strains listed were kindly provided by Dr Kirsten Rosendal Department of Diagnostic Bacteriology Statens Seruminstitut

Occasionally other microorganisms *e g* yea ts were stained but these were easy to exclude because of their differing morphology

TABLE 3

Stainability with FITC Labelled Antiserum to N gonorrhoeae
Various Unrelated Species

	Number of strains examined	Stained with FITC labelled		
		Normal rabbit serum	Anti gonococcal serum	Anti gonococcal serum mixed with unlabelled normal rabbit serum
Staphylococcus albus	101	–	\	–
Staphylococcus aureus	115	–	\	–
Staphylococcus aureus	163	\	–	–
Staphylococcus aureus	4?	++++	++++	\
Streptococcus pyogenes group A	4	+ → ++++	+ → ++++	\
Streptococcus pyogenes group B	5	\	N	N
Streptococcus pyogenes group C	4	+ → ++++	+ → ++++	\
Streptococcus pyogenes group D		+ → ++++	+ → ++++	\
Escherichia coli type O1–O?5	2	\	\	–
Proteus mirabilis	6	–	\	–
Aerobacter cloa ae		–	\	–
Haemophilus vag nalis (I apage	1	–	\	\
Pasteurella multo ida ATCC 10544	1	–	\	\
Pasteurella st ains isolated from r bbits ( )	4	–	\	\
N *gonorrhoea* (fresh control)		\	++++	++++

Except one stra n shi h shov ed a ++++ rea ti n he experiment has not yet been r peated

++++ Brilliant c psule lik fluorescence typical app a of specific staining

+ → ++++ Variati n in degre of fluore cence of bacter the same pure culture

\ Negative *i e* no or w e k flu res cence

\ Not examined

By Dr A *Reyn* reference No 36

B *Application of the FAT in the Diagnosis of Gonorrhoea*

Preliminary experiments were performed on smears of urethral discharge from male patients suspected to be suffering from gonorrhoea. On account of a brilliant non specific staining of leucocytes and occasional staining of microorganisms other than gonococci the identification of gonococci was found to be very difficult. A satisfactory reduction in the non specific staining of leucocytes was not achieved by absorption of the conjugates with various tissue powders human erythrocytes or human leucocytes in addition the specific staining of gonococci was more or less reduced. The application of different counterstaining techniques was also examined. The effect of Lissamine rhodamine RB 200 labelled albumin (40) was found to be variable and insufficient. Normal globulins labelled with either Lissamine rhodamine RB 200 or TMRITC (tetramethylrhodamine isothiocyanate (BBL)) gave a more even red background but neither of the two conjugates reduced satisfactorily the non specific staining of leucocytes. The same poor results were obtained with Evans blue (14) and Flazo Orange (18 19). With all the methods the counterstain occasionally interfered with the specific staining of gonococci giving them all shades of colour from red to yellowish green.

Ovcinnikov (33) suggested that digestion of the smears with trypsin prior to staining would solve the problem. In our hands trypsin treatment diminished not only the non specific staining of leucocytes but also reduced considerably the specific staining of gonococci.

The effect of alteration of the ratio between dye and protein during the labelling procedure in combination with chromatography on DEAE cellulose will be described in a subsequent paper. These experiments and those carried out by other authors showed that gammaglobulins purified on DEAE cellulose after labelling would never give a non specific staining of leucocytes and that this staining was caused by over labelled globulin molecules (5 13 27). The latter point explains why it was not possible to block the staining of leucocytes by the addition of unlabelled normal rabbit serum to the conjugate.

Nevertheless the diagnosis made on direct smears from discharge still remained problematic since the intracellular gonococci were never stained and the extracellular gonococci often occurred sparsely being only stained brilliantly when not covered by secretion.

The following experiments with specimens from patients were performed using a modification of *Deacon*s technique (12). Duplicate specimens were kindly supplied by the physicians at the Venereological Out Patients Department of Rudolph Berghs Hospital Copenhagen and by Dr Henning Schmidt Department of Dermatology and Venereology Rigshospitalet Copenhagen. The transportation time never exceeded 4–6 hours. One swab was used for routine culture the diagnosis being confirmed by Gram staining fermentation reactions and oxydase

reaction This routine result was available after 2–3 days at the earliest The other swab was used for preparation of two smears for undelayed FAT and for inoculation on the routine medium followed by preparation of smears for delayed FAT as described under Materials and methods

The duplicate specimens were received during two periods The results from the first period have been published previously and a few cases described in detail (26) A total of 664 duplicate specimens were examined corresponding to 170 female and 161 male patients Among 664 specimens 178 were found positive by culture and 205 positive by the FAT This corresponds to an increase of 15 per cent in positive findings by the FAT 66 male patients were found positive by both methods 65 female patients were found positive by culture against 67 by the FAT In the case of the males only specimens from the urethra were received Three specimens were taken from each female patient viz from the urethra the cervix and the rectum The results are shown in Table 4 If the FAT were used the increase in positive specimens from the three sites in the female material was about 24 per cent a major part of this yield being from the more heavily contaminated rectal specimens

TABLE 4

Identification of Neisseria gonorrhoeae in 170 Female Patients Comparison of FAT and Culture

	Patients		Urethra		Cervix		Rectum		All sites	
	FAT	culture	FAT	culture	FAT	culture	FAT	culture	FAT	culture
Pos. FAT & pos culture	64	64	44	44	56	56	7	7	107	107
Pos FAT & neg culture	3		9		6		17		32	
Neg FAT & pos culture		1		3		2		0		5
Total positive sites			53	47	62	58	24	7	139	112
Total positive patients	68		56		64		24			

During the last part of the second series the hospitals supplied an extra smear corresponding to each specimen These smears were used for Gram staining and compared with the results of the undelayed FAT By these methods a total of 48 specimens and smears from 48 male patients and 171 specimens and smears from 58 female patients were examined

Among the 48 male patients the same 30 patients were found positive both by culture and by delayed FAT Out of these 30 patients 28 were found positive by the undelayed FAT one patient was negative and from another both slides were lost The corresponding Gram stained smears showed 19 positive containing extracellular Gram nega

B *Application of the FAT in the Diagnosis of Gonorrhoea*

Preliminary experiments were performed on smears of urethral dis charge from male patients suspected to be suffering from gonorrhoea On account of a brilliant non specific staining of leucocytes and occa sional staining of microorganisms other than gonococci the identifica tion of gonococci was found to be very difficult A satisfactory reduction in the non specific staining of leucocytes was not achieved by absorp- tion of the conjugates with various tissue powders human erythrocytes or human leucocytes in addition the specific staining of gonococci was more or less reduced The application of different counterstaining tech niques was also examined The effect of Lissamine rhodamine RB 200 labelled albumin (40) was found to be variable and insufficient Normal globulins labelled with either Lissamine rhodamine RB 200 or TMRITC (tetramethylrhodamine isothiocyanate (BBL)) gave a more even red background but neither of the two conjugates reduced satisfactorily the non specific staining of leucocytes The same poor results were ob tained with Evans blue (14) and Flazo Orange (18 19) With all the methods the counterstain occasionally interfered with the specific stain ing of gonococci giving them all shades of colour from red to yellowish green

Ovcinnikov (33) suggested that digestion of the smears with trypsin prior to staining would solve the problem In our hands trypsin treat ment diminished not only the non specific staining of leucocytes but also reduced considerably the specific staining of gonococci

The effect of alteration of the ratio between dye and protein during the labelling procedure in combination with chromatography on DEAE cellulose will be described in a subsequent paper These experiments and those carried out by other authors showed that gammaglobulins purified on DEAE cellulose after labelling would never give a non specific staining of leucocytes and that this staining was caused by over labelled globulin molecules (5 13 27) The latter point explains why it was not possible to block the staining of leucocytes by the addi tion of unlabelled normal rabbit serum to the conjugate

Nevertheless the diagnosis made on direct smears from discharge still remained problematic since the intracellular gonococci were never stained and the extracellular gonococci often occurred sparsely being only stained brilliantly when not covered by secretion

The following experiments with specimens from patients were per formed using a modification of *Deacon* s technique (12) Duplicate specimens were kindly supplied by the physicians at the Venereological Out Patients Department of Rudolph Bergh s Hospital Copenhagen and by Dr Henning Schmidt Department of Dermatology and Venereo logy Rigshospitalet Copenhagen The transportation time never ex ceeded 4–6 hours One swab was used for routine culture the diagnosis being confirmed by Gram staining fermentation reactions and oxydase

The above mentioned experiments were performed on a material which in some respects must be considered as selected viz 1) The transportation time was short namely 4–6 hours and 2) the specimens were collected by experienced venereologists from a group of patients with a high incidence of gonorrhoea Normally specimens are also transported in modified Stuart medium and the transportation time very seldom exceeds 24 hours The culture method used for routine diagnosis reveals about 10 per cent positives out of 110 000 specimens per year In order to get an impression of the practicability of the FAT in the routine work 821 of the swabs received were inoculated on two plates one for the usual procedure and one for the delayed FAT The FAT was given a handicap as the medium for routine culture was always inoculated first The FAT was performed on a single smear from each culture for 502 specimens and on duplicate smears for the following 319 specimens

The 502 single smears corresponded to 91 male patients and 184 female patients Among the 91 male patients 12 were found positive by both methods and 2 by the FAT alone The 184 female patients showed 23 positive results by both methods 2 positive by the FAT alone and another 3 positive by culture alone The number of specimens from the 184 female patients was 409 with 31 positive results by both methods 7 positive by the FAT alone and 6 positive by culture alone

The 319 duplicate smears were from female patients only 149 specimens were found positive by culture and 163 by the FAT *i e* an increase of about 9 per cent

DISCUSSION

During recent times the most important applications of immunofluorescence in diagnostic bacteriology have been reviewed b *Cherry & Moody* (1) They point out that immunofluorescence of bacteria has only two dimensions—morphology and serological specificity and further lack of specificity can be tolerated only if the morphology of the cross reacting antigen clearly distinguishes it from the specific antigen In the present immunofluorescent studies of gonococci it was not found possible to fulfil these conditions The known serological cross reactivity within the genus *Neisseria* was reflected in the stainability of species other than *N gonorrhoeae* with FITC-labelled gonococcal antisera resulting in morphologically identical pictures It was possible to inhibit the weaker reactions by the addition of normal rabbit serum However the stronger reactions observed in some meningococcal strains could not be eliminated in this way Absorption of the conjugates with meningococci was effective but at the same time the degree of staining of the gonococci was diminished presumably due to the competition of a high content of soluble meningococcal antigens in the absorbed conjugates Such antigens were easily demonstrable b

the Ouchterlony gel precipitation technique (unpublished data) From a practical point of view the problem is not serious since meningococci are seldom found in specimens received for identification of gonococci A greater problem was the strong reactivity shown by about 10 per cent of all the strains of *Staphylococcus aureus* examined organisms in which the morphological differences from *N gonorrhoeae* are too delicate to be differentiated in immunofluorescent stained smears This problem has occurred to nearly all workers using FAT for bacteriological diagnosis independent of the antigen antibody system involved (1 2 6 21 27, 35) *Danielsson* (9) used absorption of the antigonococcal conjugate with a strongly cross reacting strain of *Staphylococcus aureus* It is logical to do so if the reaction can be considered as an expression of antigenic relationship or as a detection of naturally occurring antibodies According to the author s experience with this method of absorption the reduction in staining the gonococci was of a similar degree or even more pronounced than the reduction in staining the staphylococci This might be expected if the staining is considered not as a result of an antigen antibody reaction but as a result of a non specific adsorption of globulins to the surface of certain staphylococci (27 28) *This consideration is also in agreement* with the fact that the addition of a surplus of non labelled normal rabbit serum to the conjugate has proved to be an easy and efficient way of blocking the staining of staphylococci Thus although the morphology of the cross reacting antigen did *not* clearly distinguish it from the specific antigen it is possible to obtain a reliable result when working under known conditions

At the beginning of the discussion *Deacon, Peacock Freeman & Harris* (11) state the following in their first paper Our evidence appears to indicate that *N gonorrhoeae* possesses a heretofore unrecognized antigenic component similar in character to Vi antigen of *S typhi* or K antigen of the Escherichia group The new antigen appears to be *species specific and may be recognized by* slide agglutination tests employing living or formalin killed cultures and by fluorescent antibody technics This new antigen which was assumed to be associated with specific staining in the FAT as well as with the virulence of the microorganism was also associated with freshly isolated *inagglutinable* gonococcal cultures

In the above mentioned paper the authors did not distinguish between various forms of inagglutinability The inagglutinability was described in two connections 1) as *confirming* the observations of *Wilson* (42) and 2) as revealing an antigen referred to as GC—K(B) Apparently this implies a contradiction since the data published comprise only the demonstration of O inagglutinability of some recently isolated strains *Wilson* describes inagglutinability of a recently isolated strain in an antiserum against another living recently isolated

strain an observation which probably reflects a type variation and not the presence of a species specific antigen which is easily lost on subculture On the other hand an antigen of type K(B) is revealed by its O inagglutinability (41)

On account of the important properties said to be connected with inagglutinability attempts were made to isolate such inagglutinable strains Antisera against both formalin killed and heated bacteria of two recently isolated and two stock strains of N *gonorrhoeae* were available For all these strains O inagglutinability was demonstrated the antisera showed a similar degree of specificity irrespective of whether they were used in the FAT or tested in complement fixation test against antigens of different species of *Neisseria* Using antisera against formalin killed gonococci in the agglutination test 95 per cent of 201 living cultures of N *gonorrhoeae* were found to be autoagglutinable This percentage was diminished although not encouragingly when formalin killed suspensions were used Further examination of possible inagglutinability was not performed

The finding that older gonococcal cultures are poorly stained in the FAT has been confirmed by all workers in this field This does not appear to mean a loss of antigen (K antigen) since a new subculture 16–18 hours old exhibits the usual brilliant fluorescent picture as shown by smears from the primary culture In 16 subsequent subcultures three strains selected at random were followed without any change in morphology or in staining intensity *Danielsson* (8) has controlled the staining ability after up till 102 subcultures without being able to demonstrate any loss of antigenic components important for the staining reaction In other experiments (8) he finds that heat labile as well as heat stable antigens take part in the reaction in the FAT In agreement with the latter observation FITC labelled monovalent antiserum against reference strain VI (11413) has proved to be highly valuable for diagnostic purposes This strain is known to contain several heat stable antigens of *Neisseria gonorrhoeae* (37)

That this new K(B) antigen should possess characteristics similar to Vi antigen of *Salmonella typhi* has been questioned first by *Ocenni Kov* (33) and later indirectly in publications from *Deacon*s laboratory (22 23) In this laboratory the virulence of gonococci was attributed to the type of colony No agreement was found between colony type and *any* serological reaction nor between colony type and stainability in the FAT All four colony types were specifically stained by FITC labelled antiserum against the virulent type I as well as the avirulent type IV colony This excludes definitely the original conception that the staining reaction was linked to a labile virulence associated surface antigen

For diagnostic purposes the ideal fluorescent antibody technique was originally thought to be the direct staining of specimens (smears) with

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BRIEF REPORT

PERIPHERAL NERVE CHANGES IN TAY SACHS AND BATTEN SPIELMEYER VOGT DISEASE

A preliminary report

By *Krister Kristensson* *Ingve Olsson* and *Patrick Sourander*

In Sweden two well defined types of familial amaurotic idiocy i e the infantile or Tay Sachs disease (TSD) and the juvenile or Batten Spielmeyer Vogt disease (BSVD) have repeatedly been reported (cf *Feg Olofsson et al* 1966 *Sjogren* 1933 *Rayner* 1962) Recent chemical (*Svennerholm* 1963) histochemical (*Zeman & Albert* 1963 *Kristensson & Sourander* 1966) and ultrastructural (*Zeman & Donahue* 1963) investigations have shown that in spite of certain common clinical and light micro scopical features these two conditions must be regarded as separate nosological entities Although the changes in the central nervous system are well known no information exists about the state of the peripheral nerves in these diseases

In the past the study of peripheral nerves in neurolipidoses has been much ne glected However it has recently been shown that both in metachromatic (MLD) and globoid cell (GLD) leucodystrophy the peripheral nerves are severely involved probably as a result of a basic metabolic disturbance affecting the whole nervous system (*Sourander & Olsson* 1967 *Allen & de Veyra* 1967)

Considering the frequent use of peripheral nerve biopsies for the diagnosis of MLD and the recently detected peripheral neuropathy in GLD a histological scrutiny of peripheral nerves in other neurolipidoses including various types of amaurotic idiocy seems justified

Materials and Methods Autopsy material from one case of TSD and three cases of BSVD were examined Patho anatomical data concerning the central nervous system and the visceral organs in the TSD case including chemical findings have been presented elsewhere (*Feg Olofsson et al* 1966) However in this paper the peripheral nerve changes were overlooked Corresponding information about BSVD is to be published (*Kristensson Rayner Sourander Svennerholm*) Paraffin em bedded specimens from the spinal cords and the proximal part of the sciatic nerve were stained with luxol fast blue cresyl violet and impregnated with silver accord ing to Palmgren for axons Spinal nerve roots one dorsal root ganglion and the oculomotor nerve from the TSD and one dorsal root ganglion from one of the BSVD cases were treated in the same way

Results

Tay Sachs disease The motor neurons of the cord presented changes similar to those seen in the cerebral cortex (*Feg Olofsson et al* 1966) i e marked distension of the cytoplasm and of some processes indicating lipid storage The white matter of the cord showed severe destruction of the axons and myelin sheaths in the ventral and lateral tracts whereas the dorsal tracts were better preserved A moderate degenera tion of myelin sheaths and of axons occurred both in the ventral and dorsal roots Loss of neurons associated with proliferation of capsular and interstitial cells were frequently observed in the dorsal root ganglion Remaining neurons showed variable swelling of the cytoplasm

The sciatic nerve showed an almost total destruction of myelin sheaths associated

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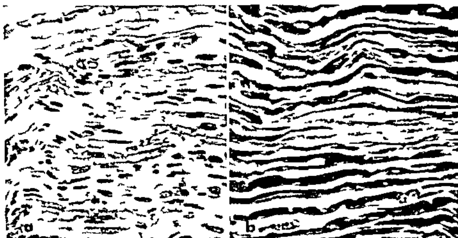


Fig 1

Sciatic nerve from the TSD case a) Luxol fast blue cresyl violet
b) Impregnation with silver for axons

with a crowding of Schwann cell nuclei (Fig 1a) One remarkable finding was the frequent occurrence of irregular elongated thickenings of the axons (Fig 1b) However compared to the severe le ions of the m elin sheaths the axonal changes were quite modest The extracerebral part of the oculomotor nerve showed changes resembling those of the sc atic nerve

Batten–Spielmeyer–Vogt disease A marked neuronal storage of lipofuscin and some loss of neurons was present in the anterior horns No obvious changes were s en in the white matter of the rd Marked loss of neurons associated with proliferation of capsular and interstitial cell were present in the dorsal root ganglion In the remaining neurons lipofuscin was stored

A very large number of the myelin sheaths were destroyed in the sciatic nerves in all cases In one of them the de truction was partly of segmental type The axons in two of the cases sho ed extensive fragmentation but in the remaining case the degeneration was only slight

Discussion

In view of the chemical differences between TSD and BSVD it is appropriate to discuss separately the peripheral nerve change observed n the e diseases

In TSD a tenfold in re e of the typi al C$_{M}$ gangli side in the pinal roots ha been detected by S ennerh lm (*Feg Olofsson et al* 1966) Since not only the perikaryon of neuron but als the axons in th central nervous system show swellings due to gangliosid t r the p ssib lity exi ts that the th kened a ons f the peripheral nerv i d t n in r sed content of gangli ide I is not possible to decide whether th v t n at on of the myelin sheath s s second r to a disturbed relation b [l t n th m elin and the axon or nduc d by alte ed metabol m f the S h i ll

Vari u expla t f tl erc degeneration of nerv fibres in BSVD an be considered Th l t n b th f xons and myelin l e th p ul l in two of the case ma i l t the degene tion of neur n tn n r or horns of the spinal c i i tl d al o t ganglia or it m l e pr n i h n rit onal f ctors during th i r ng t m nal stag of the d eas St ll th po sib lity remains that the i h l n r h nges are connected with a b si metal lic disturbance The n t n al ut the patho enesis of th per ph r l ner c l ions in TSD and BSVD n n t b an wered b way of conv ntional m thod but call for further investig n in lu i ng h stochem try and lectron mi ro copy applied to biop y materi l fr n ri u ta es in TSD and BSVD

Summary

Previously unrecognized peripheral nerve changes in one case of Tay Sachs disease and in three cases of Batten-Spielmeyer-Vogt disease are described and discussed

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BRIEF REPORT

ISOLATION OF ADENOVIRUS FROM A SKIN VESICLE AND CEREBROSPINAL FLUID FROM A PATIENT SUFFERING FROM STEVENS JOHNSON SYNDROME

By Pirkko Pohjanpelto and O Pettay

Several factors including administration of long lasting sulpha preparations and infection with herpes simplex or mycoplasma have been connected with Stevens Johnson syndrome (1) Demonstration of rise in complement fixing antibodies against adeno group antigen in two patients suffering from Stevens-Johnson syndrome suggest that also adenoviruses may in some case play a role in manifestation of this syndrome (2) In this paper a report is given of isolation of adenovirus type 3 from a skin vesicle and cerebrospinal fluid from a patient hospitalized on account of Stevens Johnson syndrome

On the suspicion of urinary infection the patient a girl of five years received nitrofurantoin and sulpha metoxipyridazine Nine days after the first administration of sulpha preparations the patient developed a high fever 39 C and a few days later vesicles appeared both in the mucous membranes and the skin The patient was somnolent and sometimes unorientated The cerebrospinal fluid was clear it contained 15 mg protein 90 mg% glucose and one cell/µl

From a skin vesicle and from the cerebrospinal fluid an agent was isolated that caused degeneration of U cells (continuous human amnion cell line) fixed complement in the presence of adenovirus immune serum and agglutinated red cells from a chimpanzee Haemagglutination was inhibited by type 3 adeno immune serum The titre of complement fixing adeno antibodies in the serum of the patient was 1 64 Any rise in the titre of the antibodies was not demonstrated in the course of the illness

Isolation of adenovirus type 3 from a skin vesicle and from the cerebrospinal fluid in a patient suffering from Stevens Johnson syndrome is in support of the hypothesis that adenoviruses in some cases may play a role in the aetiology of this syndrome

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Immunology

Nilsson U R Institute of Medical Microbiology University of Lund Sweden and Scripps Clinic and Research Foundation La Jolla Calif U S A STUDIES ON THE MODE OF ACTION OF THE FIFTH SIXTH AND SEVENTH COMPONENTS OF HUMAN COMPLEMENT (C) IN IMMUNE HEMOLYSIS

The classical third component of C is presently considered to consist of at least six different factors C'3 C5 C6 C7 C'8 and C'9 These studies concerned the mode of action of C'5 C6 and C7 As a group these components were found to interact with sensitized sheep erythrocytes (EA) which have reacted with the first fourth second and third components of C EAC 1a 4 2a 3 The reaction product was designated EAC'1a 4 2a 3 5 6 7

Kinetic analysis indicated the reaction sequence C5–C6–C7 Isolation of the intermediate complexes corresponding interaction of EAC 1a 4 2a 3 with C5 or with C5 and C'6 at physiological ionic strength were unsuccessful Formation of EAC 1a 4 2a 3 5 6 7 proceeded only when C5 C'6 and C7 were present simultaneously in the reaction mixture This interdependence between the three components suggested fluid phase interaction Protein protein interaction was demonstrated between C5 and C6 and C5 and C'7 by density gradient ultracentrifugation Dose response studies furnished support for the hypothesis that C5 C'6 and C'7 constitute a functional unit

This work was done in collaboration with Dr H J Muller Eberhard at Scripps Clinic and Research Foundation La Jolla Calif

Holm G & Johansson Bo the Wenner Gren Institute Stockholm IN VITRO STUDIES OF LYMPHOCYTES FROM PATIENTS WITH HODGKIN'S DISEASE AND CHRONIC LYMPHATIC LEUCEMIA

To be published in Clinical Experimental Immunology 1967

Magnusson Pernilla & Kallings L O The Department of Virology Karolinska Institutet and the Department of Bacteriology National Bacteriological Laboratory Stockholm ANTIBODY SPECTRUM OF HUMAN GAMMA GLOBULIN FOR CLINICAL USE

Human gammaglobulin is widely used for prevention and treatment of a vast variety of infectious conditions To estimate the value of this administration it was found imperative to have a better knowledge of the microbiological spectrum of antibodies present and to what extent the different batches of gammaglobulin are

similar in composition In this study 10 batches of human gammaglobulin have been tested for the ability of neutralizing an assortment of infectious agents generally occurring in Sweden It is also speculated on whether gammaglobulin representing a great number of blood donors could be used for a rough epidemiological screening A more comprehensive report on this study will be presented elsewhere

Jonsson Jonas & Fagraeus Astrid Department of Immunology National Bacteriological Laboratory Stockholm THE MIXED HAEMADSORPTION TECHNIQUE AS A DIAGNOSTIC TEST FOR THYROID ANTIBODIES

If circulating thyroid antibodies have a pathogenetic effect by damaging living thyroid cells they must in the first place be assumed to exert this effect by attacking the cell membranes The mixed haemadsorption technique applied to monolayer cultures of thyroid epithelial cells (1) has proved to be a convenient method for the demonstration of such antibodies especially in large scale examinations Sera from 259 cases of thyroid disease 34 cases of connective tissue disease and 347 apparently healthy controls were examined with this test A reaction in the serum dilution 1/1600 or more indicated a diagnosis of chronic thyroiditis or thyrotoxicosis with a probability of 80 per cent The probability that a reaction at this titre level should occur in the absence of thyroid disease was 6 4 per cent The mixed haemadsorption reactions obtained were of two types called ring zone and filled zone reactions Ring zone reactions were obtained with 2/3 of the sera from cases of chronic thyroiditis 1/3 of the sera from cases of thyrotoxicosis less than 10 per cent of the sera from other thyroid diseases and with 3 per cent of the control sera There was a strong correlation between the ring zone reaction and a reaction with cytoplasmic antigen in the immunofluorescent test The organ specificity of the reaction was confirmed by tests with other types of human cell cultures and by absorption experiments The incidence of thyroid antibodies in patients sera as well as in the control sera varied with age and sex in the same way as in the common standard tests for thyroid auto antibodies The thyroid antibodies demonstrated by the mixed haemadsorption technique thus have the same diagnostic implications as those which are demonstrated by the immunofluorescent test and the complement fixation test The results reported above suggest that the antigen antibody system that gives rise to the ring zone reaction is more specific for chronic thyroiditis than others Compared to the diagnostic tests employed so far the mixed haemadsorption test seems to increase the possibilities to differentiate serologically between chronic thyroiditis thyrotoxicosis and atoxic colloid goiter

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Lundbeck H & Tirunarayanan M O National Bacteriological Laboratory Stockholm ANTIBODIES AGAINST LIPASE FROM STAPHYLOCOCCI

A method for determination of staphylococcal lipase with the aid of egg yolk agar plates has been worked out and published previously The enzyme suspension to be tested is filled into a well cut out in the agar and allowed to diffuse into the agar at 37 C. Lipase activity is observed as a clarification and precipitation zone surrounding the well The diameter of the zone can be used to quantitate the lipase activity The reaction is inhibited by sera containing antibodies against lipase A

technique for determination of antilipase antibodies with the aid of standardized quantities of lipase has been worked out This technique was used to demonstrate the o currence of antilipase antibodies in human sera from different age groups and to study the correlation between antilipase and antistaphylolysin antibodies It was found that the level of antibodies against lipase decreased with age from 0 to 6 months indicating the presence of maternal antibodies and increased with higher age reaching a constant level in groups above 10 years There was a good correlation between antilipase and antistaphylolysin titers The antilipase activity was localized in the 7S gammaglobulin fraction of serum

Wide L & Porath J Department of Clinical Chemistr University Hospital Uppsala and Institute of Biochemistry University of Uppsala
A RADIO IMMUNOLOGICAL METHOD FOR DETERMINATION OF PROTEINS WITH THE USE OF SEPHADEX COUPLED ANTIBODIES

A new modification of the radioimmunoassays for proteins was presented (*L Wide & J Porath* Biochim Biophys Acta 130 (1966) 257-260) The immunoglobulins in an antiserum were precipitated by ammoniumsulphate and chemically coupled to an insoluble polymer a Sephadex derivate (isothiocyanatophenoxyhydroxypropyl Sephadex) prepared a cording to Axén and Porath (*R Axén & J Porath* Nature 210 (1966) 367) The immunosorbent thus prepared may be freeze dried The antigen binding capacity of the antibody Sephadex particles was not altered after 12 months storage at + 4 C. One gram antibody Sephadex particle could be used for more than 10 000 reactions In the radioimmunoassay the sample to be investigated the antigen labelled with a radioactive isotope (I) and the antibody Sephadex were added to a testtube and incubated for 24 hours by slowly rotation around a horisontal axis The antibody bound labelled antigen was separated from the unbound by centrifugation The radioactivity in the Sephadex precipitate was counted in a scintillation detector The sensitivity of the assays could be increased by preincubation of the antibody Sephadex with the solution to be investigated prior to the addition of the labelled hormone and incubation for several days In this way less than 1 pg of luteinizing hormone per ml solution could be detected The technique has hitherto been applied to the assay of insulin growth hormone chorionic gonadotrophin luteinizing hormone and immunoglobulin C The high sensitivity precision and simplicity of this method make it as suitable for scientific purposes as for routine hospital laboratory use

Britton S Institute of Tumor Biology Karolinska Institutet Stockholm STUDIES ON THE PERSISTENCE OF IMMUNOGENICITY OF SOLUBLE AND PARTICULATE ANTIGENS IN THE MOUSE

The persistence of immunogenicity in the mouse of sheep red blood cell antigens and bacterial lipopolysaccharide antigens of *F c* / 0 ? B5 was determined utilizing a transfer system Mice were immunized with the respective antigen and after different time intervals the mice were lethally irradiated and repopulated with syngeneic normal lymphoid and bone marrow cells The induction of a primary antibody response in the transferred cell population indicated the retained immunogenic material in the recipient. In this system it was shown that the immunogenicity of SRBC antigens persisted for 14 [illegible] whereas the [illegible] antigen persisted for more than 45 days

A protein antigen human serum albumin (HSA [illegible] was studied in a modified tran

fer system Utilizing the notion that soluble HSA does not induce a primary response in the mouse but triggers a secondary mice were injected with small amounts of soluble HSA and after different time intervals injected with preimmunized syngeneic spleen cells The induction of a secondary antibody response would indicate retained immunogenic HSA The half life of immunogenicity of HSA measured by this method and the half life of radioactively labelled HSA in serum of the normal mouse did not differ statistically from each other

Virology

Norrby E & Wadell G Department of Virus Research Karolinska Institutet Stockholm SOLUBLE COMPONENTS OF ADENOVIRUS TYPE 4

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Svehag S E & Bloth B Department of Immunology National Bacteriological Laboratory and Department of Virus Research Karolinska Institutet Stockholm EXAMINATION OF VIRUS GAMMA M ANTIBODY COMPLEXES BY ELECTRON MICROSCOPY 1 CONFIGURATION OF GAMMA M ANTIBODIES AND MODE OF ATTACHMENT TO ANTIGEN

Poliovirus γM antibody complexes were examined by the negative staining technique in the electron microscope The virus was purified by equilibrium centrifugation and rabbit γM antibodies by zone centrifugation or gel filtration The specificity of the poliovirus γM antibody reaction was established by the use of heterologous antigen and γM fractions from normal sera N and H virus particles aggregated separately in the presence of γM antibodies against both these antigens

The size of specific poliovirus aggregates and the density of the cross linking network of γM antibodies increased with increasing relative γM antibody concentrations Employing intermediate or rather high relative antibody concentrations single virions or virions in the periphery of aggregates were often surrounded by an aura of loops The maximal length of the turn of the loops was 220 to 250 A and their width 35 to 40 A suggesting that they represented γG like 7S subunits of the γM molecule The maximal number of subunits contained in one γM molecule appeared to be 5 or 6 The distribution of the lengths of γM antibodies bridging virions had a mode and median of about 330 A and maximal value of 360 to 370 A

Biberfeld Gunnel & Fagraeus Astrid National Bacteriological Laboratory Stockholm EARLY SEROLOGIC DIAGNOSIS OF RABIES

Antibodies were demonstrated in rabiesinfected dogs during the incubation period Five dogs were inoculated intramuscularly with street virus (Dr Schmidt Atlanta) and blood specimens were collected every third day The dogs fell ill 10–17 days after inoculation and died 1–5 days later Antibodies against rabies were tested with fluorescent antibody (FA) and mixed haemadsorption (MH) technique in this laboratory and by serum neutralization (SN) by Dr Schmidt The antigen for the FA test consisted of green monkey kidney (GMK) cells infected with rabiesvirus (Flury HEP HDCS) and centrifuged directly on to slides in a special rotor After fixation in aceton the antigenpreparations could be stored at —20 C for at least two months FA antibodies (titres 1/10–1/40) were demonstrated in 4 of 5 dogs 4 10 days before onset of illness and in the fifth dog on the day of clinical onset Four

of the dogs also developed MH and SN antibodies No antibodies were found 1–3 days after inoculation

Using a fluoresceinconjugated antidog gamma globulin serum rabies antibodies were also demonstrated in a fox injected with two doses of live Flury HEP vaccin

In addition a rise in FA antibodies from < 1/5 to 1/80 was demonstrated in a patient given a full treatment with sheep brain vaccin (Lister institute)

Tirunarayanan M & Lundbæk H National Bacteriological Laboratory Stockholm RELATIONSHIP OF pH TO SYNTHESIS OF STAPHYLOCOCCAL TOXINS AND ENZYMES

A strain of *Staph aureus* was grown in a glass tank under constant pH conditions Nutrient broth containing 1 per cent glucose was used The bacteria acted upon the glucose during growth and liberated acid which was titrated to a definite pH with $KHCO_3$ using an automatic pH titration assembly and thus the cultures could be maintained at any desired pH for 24 hours Analysis for the different toxins and enzymes in the culture supernatants indicated that coagulase production was optimal at pH between 7 5 and 8 0 α hemolysin was produced around pH 6 2 and hyaluronidase at pH 6 5 The production of two phosphatases was demonstrated one at pH 8 0 and another at pH 5 5 These studies emphasize the importance of pH in the production of the extracellular enzymes and toxins and the careful consideration that must be given to the pH when comparing different biologic properties of clinical strains

Bacteriology

Löfström G Hallander H O & Laurell G Department of Medical Microbiology Division of Bacteriology University of Uppsala Uppsala PRODUCTION OF STAPHYLOCOCCAL HAEMOLYSINS STIMULATED BY PENICILLIN IN LOW CONCENTRATION

In previous papers (1 2) it was shown that penicillin G and methicillin *in vitro* stimulated the production of staphylococcal haemolysins The dermonecrotic effect of staphylococci was also enhanced in rabbits which had received moderate intramuscular injections of penicillin

Some other antibiotics were tested *in vitro* Chloramphenicol acted like penicillin and stimulated in low concentrations the production of staphylococcal haemolysin Streptomycin and the tetracyclines showed no such activity

Separation in a Sephadex G 100 column was performed on supernatants of cultures which had grown in the presence of 0 1 IU/ml of penicillin (I) or without penicillin (II) It is known (3) that two active fractions appear one within 25–30 per cent of the column volume containing mainly delta lysin the other within 45–6 per cent of the volume containing mainly alpha lysin The experiment showed that the major part of the haemolysin ... belonged for culture I to the first fraction for culture II to the second fraction That may indicate that the addition of the antibiotic stimulated the production of alpha lysin while the production of delta lysin was probably inhibited by the presence of penicillin The results are preliminary

At the VII th International Congress for microbiology in Stockholm 1958 *Luckey* (4) advocated the theory a general validity that small amounts of agents which are in larger doses toxic make the cells ready to an enhanced production of energy

enzymes and building material This leads to an abnormally fast growth when the stressing agent does not exceed the bounds of toxicity Applied on antibiotic therapy this should mean that antibiotics in low concentration might stimulate the tissue cells to enhanced activity but also give the present bacteria some stimulus A quite delicate interaction between the cells and the bacteria is likely to occur if this theory has this general validity

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1 *Hallander H O Laurell G & Lofström G* Stimulation of staphylococcal haemolysin production by low concentrations of penicillin Acta path et microbiol scandinav 68 142–148 1966
2 *Hallander H O Laurell G & Löfström G* Enhancement of staphylococcal pathogenicity in the presence of penicillin Acta path et microbiol scandinav 68 463–464 1966
3 *Hallander H O* Fractionation of staphylococcal toxins by gel filtration Acta path et microbiol scandinav 59 Fasc 4 Separatum 1963
4 *Luckey T D* Modes of action of antibiotics in growth stimulations Recent progress in microbiology VII Int Congress for Microbiology Almqvist & Wiksell Stockholm 1958

Wickman Kristina Central Laboratory of Clinical Bacteriology Karolinska Sjukhuset Stockholm COMPETITIVE INTERACTIONS BETWEEN BACTERIAL SPECIES IN EXPERIMENTAL BURNS IN THE GUINEA PIG

The intention of the experiments was to show that when bacteria of a low pathogenicity are introduced into fresh burns they can in some case prevent or reduce colonisation by microorganisms of a higher pathogenicity which are introduced into the burns at a later stage or which occur spontaneously

Guinea pigs were used and were given thermal full skin thickness burns in the flank corresponding in size to ? per cent of the body area. A DNase and coagulase negative staphylococcus was taken from the animals normal flora and applied to the burn Similarly burned but untreated guinea pigs were used as controls *Staphylococcus aureus* of a strain which is easily identifiable because of its phage type and erythromycin resistance was later introduced into the burn by means of a spray In some series no such test strain was added but records were kept of *Staphylococcus aureus* spontaneously occurring in the wounds In all series cultures taken from the wound secretions showed a statistically strongly significant difference in both quality (the number of specimens containing Staphylococcus aureus) and quantity (the amount of *Staphylococcus aureus* measured by colony count) Of the specimens taken from the animals treated with the interfering strain 36 per cent contained *Staphylococcus aureus* against 60 per cent in the control animals The amount of *Staphylococcus aureus* in the wound secretion was estimated by counting the number of colonies per plate The were given the numbers 0–4 according to a logarithmic scale The experimental animals had an average of 0 8 against 1 9 in the control group

Lincoln K Department of Clinical Bacteriology University of Goteborg Goteborg O ANTIGEN GROUPING OF E COLI STRAINS AND DETERMINATION OF AGGLUTINATING ANTIBODIES IN HUMAN SERUM PERFORMED IN PERSPEX PLATES

Direct bacterial agglutination was performed in perspex haemagglutination plates with 8×10 cavities 0 05 ml of antigen suspension plus 0 05 ml of serum dilution

was incubated at 50 C for 4 hours left at r om temperature over night and read with a dissecting microscope at 16× magnificati n against a black background

1123 strains of *F coli* isolated from urine were investigated with 8 antisera 545 (48 5 per cent) were agglutinated 337 (30 0 per cent) were not specifically agglutinated and 241 (?1 5 per cent) were in a rough phase and agglutinated spontaneously The specifically agglutinated strains were distributed as follows

O antigen group	02	04	06	01	07	075	018	08
Per cent of total of strains	11 ?	9 8	7 0	5 6	5 3	5 1	3 0	1 4

In the serum of normal adults (blood donors and pregnant women in antenatal care) the following median titers of agglutinating coli antibodies were found

F coli O antigen	01	0?	04	06	07	08	014	018	075	90"
Reciprocal of titer	?40	320	160	80	3?0	3?0	80	80	80	640

90 was a polyvalent antigen composed of equal amounts of the nine different monovalent O antigens The highest and lowest titers noted were 1/?5?0 and 1/?0 There was no difference between men and women

Ericson Carl & Torp A Institute of Clinical Bacteriology General Hospital Malmo

INACTIVATION OF CHLORAMPHENICOL RESISTANT ENTEROCOCCI

Using a disc diffusion technique for determination of bacterial susceptibility to antibiotics it was noted that sensitive staphylococci grew up to the chloramphenicol disc in the presence of enterococci resistant to the drug

Inactivation of chloramphenic l b resistant strains has been reported for staphyloco ci (*Chabbert & D bruge* 1956) and enterobacteria (*Miyamura* 1964) but as far as known not for enteroco ci

With a modified Gots method 63 consecutively isolated chloramphenicol resistant strains of entero o ci (MIC > 30 mcg/ml) were f un l to sup p rt satellite growth of indicator organisms in contrast to more sensitive strains (MIC < 10 mcg/ml)

The inactivation mechanism remains obscure *Dunsmoor et al* (19?3) failed to show changes in the molecular structure of chloramphenicol

No stu lies seem to have been made on the clinical significance of chloramphen ol inactiv tion In the lab rator it may cause fal e recording of resistance with th disc diffusion technique (*W al ru rth* 1966) This i especially tru for cultures mixed with resistant enterococci whose colonies are easily hidden by overgrowth

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Sjöl erg I & I i th rg A A D partment of Bact r l & N tion l Ba teriological Labo at St kh lm

PHAGE AND PYOCINE TYPING OF P EUDOMONAS AERUGINOSA

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